# The effects of arterial shear on MAP kinase activation and expression of pro- and anti-inflammatory molecules in cultured porcine endothelial cells

Dissertation for the *cand.pharm* degree

**by Øyvind Ruud**


National Heart and Lung Institute
Cardiovascular Sciences Unit
Imperial College
London

and Department of Pharmaceutical Biosciences
Faculty of Mathematics and Natural Sciences
School of Pharmacy
University of Oslo
2007

# TABLE OF CONTENTS

## ACKNOWLEDGEMENTS

I would like to thank my excellent supervisor Paul Evans for his professionalism, advice and guidance as well as his uplifting presence.

Further, I would like to thank my in-house supervisor at the School of Pharmacy, University of Oslo, Harald Thidemann Johansen, for making it possible for me to come to Imperial College.

I am grateful to Mr Mustafa Zakkar (a cardio-thoracic surgeon in our group) who supervised many of the experiments performed in this project. Mr Zakkar provided the data that validated the phenotype of porcine endothelial cells (shown in Figure 3.1) and also examined the responses of porcine endothelial cells to TNFα (shown in Figure 3.2).

A special thanks to Karine Enesa for help with the confocal work, and along with Hera Chaudhury and Le Anh Luong for all the guidance they have given me.

And finally, thanks to all my colleagues in the NHLI Cardiovascular Scieneces department at Hammersmith Hospital.

October 2007

Øyvind Ruud

## ABSTRACT

Vein grafting is complicated by high rates of stenosis due to the development of vascular inflammation, intimal hyperplasia and accelerated atherosclerosis. In contrast, arterial grafts are relatively resistant to these processes. Vascular inflammation is regulated by MAP kinases (including JNK and p38) and NF-κB signaling pathways which trigger endothelial expression of adhesion molecules (e.g. E-selectin, VCAM-1) and chemokines (e.g. IL-8, MCP-1).

Blood flow over lumenal surfaces generates haemodynamic forces. Changes in flow have profound effects on the physiology of endothelial cells. This may influence disease processes that are associated with perturbations in flow, e.g. intimal thickening. Here we examined the effects of acute arterial shear stress (12 dynes/cm$^2$) on pro-inflammatory activation of porcine aortic endothelial cells (PAEC) or porcine jugular vein endothelial cells (PJVEC). We also examined whether induction of anti-inflammatory transcripts accompanied the induction of pro-inflammatory mRNA.

PAEC and PJVEC monolayers were plated onto gelatin-coated glass slides and cultured for 48h before experimentation. Cultures were then placed in a vacuum-held, parallel-plate flow chamber (Figure 2.2). Fluid was circulated through the chamber via a flow loop that held a constant flow rate, so that the shear stress imposed on the endothelial cells layer was 12 dynes/cm$^2$ (equivalent to arterial flow) (Figure 2.1). Cells were cultured in static conditions or exposed to acute shear or TNFα (10ng/ml) for two and four hours or thirty and ninety minutes to look at gene expresssion and MAP kinase activation

respectively. Transcript levels were quantified using reverse transcription, real-time quantitative PCR. The amount of each target gene was normalized by measuring cyclophilin mRNA levels. The effect of shear stress on pro-inflammatory MAP kinases and NF-κB was assessed by western blotting for a phosphorylated form of JNK and p38, and nuclear translocation of p65.

We observed that arterial shear stress induced high levels of IL-8, MCP-1 and E-selectin transcripts in PJVEC whereas PAEC were relatively resistant to the pro-inflammatory effects of shear stress (Figure 3.3 and Figure 3.4). To investigate the potential mechanism underlying the differential responses of PJVEC and PAEC we examined the effects of shear stress on the activation of NF-κB, JNK and p38. We observed that shear stress induced prolonged activation of JNK and p38 in PJVEC but only transient activation in PAEC. In contrast, the kinetics of NF-κB activation in response to shear were similar in both cell types (Figure 3.6 and Figure 3.7). Thus prolonged activity of MAP kinases may explain the hypersensitivity of PJVEC to shear stress mediated activation. Finally, we observed that anti-inflammatory transcripts (MKP-1 and XIAP) are induced by shear stress in PAEC but not in PJVEC (Figure 3.11), thus PAEC may resist pro-inflammatory activation by shear stress through inhibition of MAPK by molecules such as MKP-1 and XIAP. We suggest that the hypersensitivity of venous EC to the pro-inflammatory effects of shear stress may partly explain the susceptibility of vein grafts to inflammation and accelerated atherosclerosis.

## ABBREVIATIONS

| | |
|---|---|
| AP-1 | activating protein-1 |
| ATF2 | activating transcription factor 2 |
| BMK1 | big MAP kinase 1 |
| BSA | bovine serum albumin |
| cDNA | complementary DNA |
| COX-2 | cyclo-oxygenase-2 |
| DTT | dithiothreitol |
| EC | endothelial cell |
| ECGF | Endothelial cell growth factor |
| ERK | extracellular signal-regulated protein kinases |
| HBSS | Hanks' balanced salt solution |
| HRP | horseradish peroxidase |
| HUVEC | human umbilical vein endothelial cell |
| ICAM-1 | intercellular adhesion molecule-1 |
| IH | intimal hyperplasia |
| IHC | immunohistochemistry |
| IL-1 | interleukin-1 |
| IL-12 | interleukin-12 |
| IL-6 | interleukin-6 |
| IL-8 | interleukin-8 |
| IP-10 | interferon-γ inducible protein-10 |
| JDP | jun dimerization protein |
| JNK | c-Jun NH2-terminal kinase |
| Keap-1 | kelch-like ECH-associated protein 1 |
| LSM | laser scanning microscopy |
| MAPK or MAP kinase | mitogen-activated protein kinase |
| MAPKK or MAPK kinsase | MAP kinase kinase |
| MAPKKK or MAP3K | MAP kinase kinase kinase |

| | |
|---|---|
| MCP-1 | monocyte chemoattractant protein-1 |
| MKP-1 | MAP-kinase phosphatase-1 |
| NF-κB | nuclear factor-κB |
| Nrf2 | NF-E2-related factor-2 |
| PAEC | porcine aortic endothelial cell |
| PBS | phosphate buffered saline |
| PCR | polymerase chain-reaction |
| PJVEC | porcine jugular vein endothelial cell |
| Pro | proline |
| RHD | Rel homology domain |
| RT-PCR | reverse transcription polymerase chain-reaction |
| SAPK | stress activated protein kinase |
| SDS | sodium dodecyl sulphate |
| SMC | smooth muscle cell |
| TAK1 | transforming growth factor-β activated kinase 1 |
| Thr | threonine |
| TLR | toll-like receptor |
| TNFα | tumor necrosis factor α |
| TRAF | tumor-necrosis-factor-receptor-associated-factor |
| Tyr | tyrosine |
| VCAM-1 | vascular cell adhesion molecule-1 |
| XIAP | X-chromosome-linked inhibitor of apoptosis protein |

# 1. INTRODUCTION

## 1.1. Inflammation

Inflammation is a localised physiological response to injury or infection characterised by redness, swelling, warmth, pain and altered function. Its purpose is to neutralise damaging agents and repair damaged tissues. Acute inflammation occurs within seconds, minutes or hours whereas chronic inflammation is characterised by a prolonged time course, sometimes provoked by resistance of the harmful agent.

Acute inflammation is a complex prosess in which a variety of events contribute, starting with vascular dilatation, which enhances blood flow to tissues. This is followed by endothelial activation which is characterised by expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), E-selectin and intercellular adhesion molecule-1 (ICAM-1) which regulate binding of immune cells. Further, the activation of integrins on leukocyte surfaces allows their adherence to activated endothelium via interactions between integrins and endothelial adhesion molecules. This step facilitates subsequent transmigration of leukocytes into underlying tissues in a process known as the "adhesion cascade".

Chronic inflammation is a continuum of acute inflammation that occurs when the causative stimulus of the inflammatory response is maintained or because of aberrations in the immune system. It causes concomitant tissue destruction and repair and is involved in the pathogenesis of many diseases, such as rheumatoid arthritis and atherosclerosis.

### 1.1.1. Biology of the blood vessel wall

The blood vessel wall consists of three layers: the intima, the media and the adventitia (Figure 1.1). The innermost layer is the intima, which is comprised of a layer of endothelial cells (EC) lying on a thin layer known as the internal elastic lamina. The medial layer consists of smooth muscle cells (SMC) and extra cellular matrix supported by the external elastic lamina. This medial layer is generally thicker in arteries than in veins. Outermost is the adventitial layer which contains fibroblasts, extra cellular matrix and nerves (Mitra *et al.*, 2006).

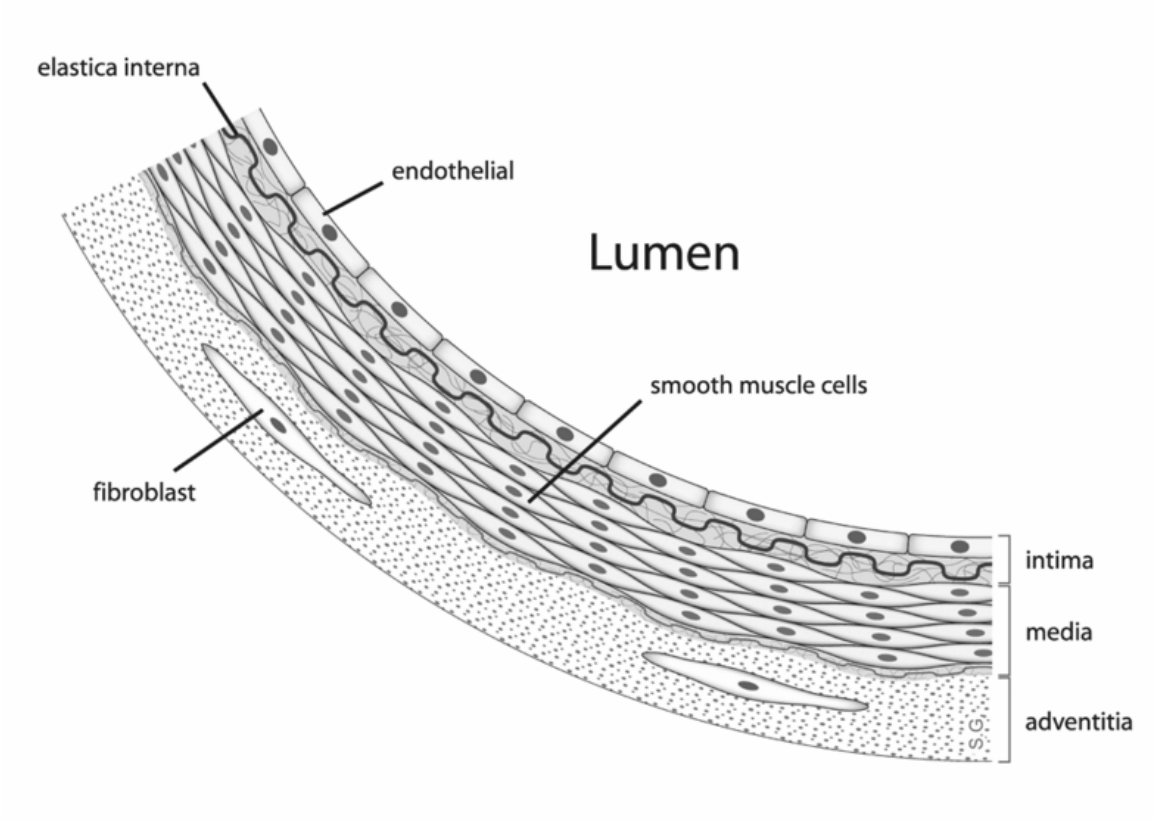


**Figure 1.1 Anatomy of an artery** (Internet reference 1)

### 1.1.2. Adhesion of leukocytes to activated endothelial cells

Endothelial cells line the interior surface of the blood vessels (Figure 1.1). The endothelium is more than a static barrier between the circulating blood and the rest of the vessel wall, it is a dynamic structure that adapts rapidly to changes in the surrounding environment. The endothelium plays a key role in regulating important physiological functions like blood pressure, blood clotting and inflammation.

The recruitment of monocytes in blood to sites of inflammation is the first step in their passage into inflamed tissues. This process is mediated by the adhesion cascade, which is comprised of a series of adhesion and activation steps (Springer, 1995) (Figure 1.2). Thus, selectins are largely responsible for initial capture of leukocytes from the blood stream, and also mediate subsequent rolling on the endothelial surface. Activation of rolling leukocytes by chemoattractants leads to increased avidity and/or affinity of leukocyte integrins and the arrest of leukocytes on the endothelial surface, followed by their transendothelial migration into the extravascular space. The capacity of EC to support interactions with leukocytes is stimulated in inflammation by the induction of numerous proteins involved in the adhesion cascade, including adhesion molecules (e.g. E-selectin, VCAM-1, ICAM-1), cytokines (e.g. tumor necrosis factor α (TNFα), interleukin-1 (IL-1)) and chemokines (e.g. monocyte chemoattractant protein-1 (MCP-1), interleukin-8 (IL-8)).

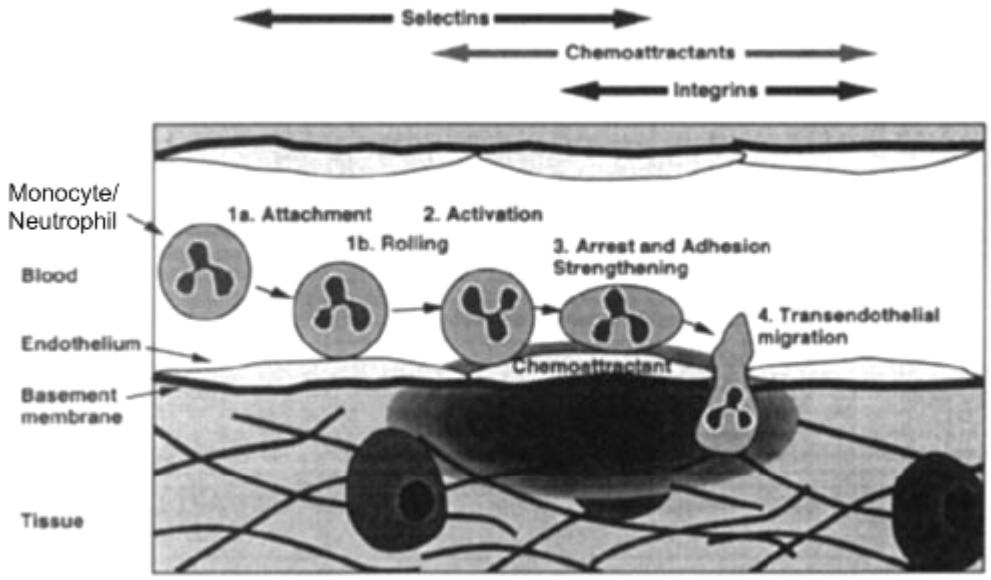


**Figure 1.2 Adhesion and arrest of cicrulating leukocytes** Adapted from (Springer, 1995)

## 1.2. Pro-inflammatory mediators

### 1.2.1. Chemokines

The chemokines MCP-1 and IL-8 are important mediators of inflammation. MCP-1 attracts monocytes such as eosinphils and basophils and T-lymphocytes, whereas IL-8 is known to attract neutrophiles and basophiles to the site of inflammation (Springer, 1995). IP-10 (interferon-γ inducible protein 10) is known to have monocyte attractant properties as well as to contribute to leukocyte adhesion to endothelial cells.

### 1.2.2. Selectins

The selectin family of proteins consist of E-selectin, P-selectin and L-selectin.

They are membrane bound adhesion molecules which upon cell activation will be expressed on the cell surface, and in turn bind to circulating leukocytes, thus contributing to vascular inflammation. E-selectin is solely expressed on endothelial surfaces and it is transcriptionally silent in quiescent EC. Upon endothelial activation by cytokines such as TNFα and IL-1 (Wadgaonkar *et al.*, 2004) or acute flow (Burns and DePaola, 2005), the transcription of E-selectin is induced. P-selectin however, is expressed on both blood platelets and in endothelial cells, where they are preformed and stored in Weibel-Palade bodies. Upon EC activation, P-selectin is rapidly mobilized to the plasma membrane to bind monocytes and neutrophils (Springer, 1995). L-selectin is expressed on all circulating leukocytes, except for a subpopulation of lymphocytes (Springer, 1995).

### 1.2.3. Other adhesion molecules

VCAM-1 and ICAM-1 are adhesion molecules that play essential roles in the capture and arrest of circulating leukocytes to vascular endothelium. VCAM-1 and ICAM-1 has been shown to be expressed in response to cytokine stimulation and acute flow (Burns and DePaola, 2005; Morigi *et al.*, 1995a; Morigi *et al.* 1995b; Nagel *et al.*, 1994).

**PRO-INFLAMMATORY SIGNALLING PATHWAYS AND THEIR REGULATION**

## 1.3. Mitogen activated protein kinases (MAPK)

MAP kinases are among the most ancient signal transduction pathways and are used to regulate a vide range of physiological processes in the immune response (Dong *et al.*, 2002). The MAPK-family consists of four MAP kinases; the extracellular signal-regulated protein kinases (ERK), the p38 MAP kinases, the c-Jun $NH_2$-terminal kinases (JNK) and the big MAP kinase 1 (BMK1, sometimes known as ERK5). (Zarubin and Han, 2005). The MAPK's are all activated by dual phosphorylation of Thr-X-Tyr residues, where X is a distinct amino acid for each MAPK member. The activaton of the MAPKs are mediated through MAPK kinases (MKKs), and in turn, the MKKs are phosphorylated by the serine/threonine kinases termed MKKKs (M3Ks) (Kumar *et al.*, 2003). Thus the MAPKs are activated by a kinase signaling cascade (Figure 1.3).

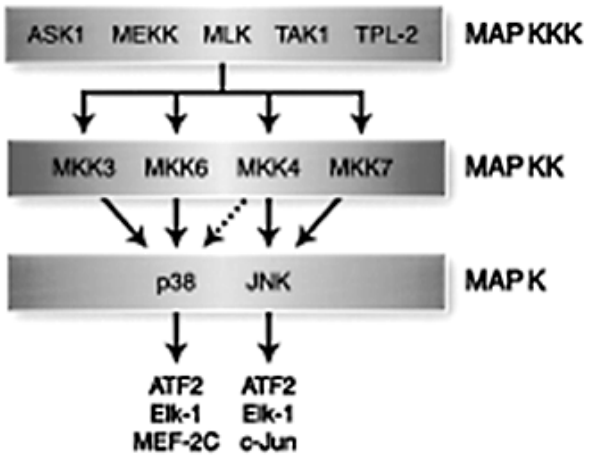


**Figure 1.3 Stress-Activated MAPK Signaling Modules** The JNK and p38 MAPK are activated by dual phosphorylation on Thr and Tyr caused by members of the MAPKK group of protein kinases. The MAPKK are activated, in turn, by phosphorylation mediated by a group of MAPKKK. Stress-activated MAPK signaling modules can be created through the sequential actions of a MAPKKK, a MAPKK, and a MAPK (Davis, 2000).

It is known that the ERK 1/2 and BMK1 are activated by mitogenic and proliferative stimuli (Dong *et al.*, 2002 and Hoefen and Berk, 2002), wheras the JNKs and p38 MAPKs are activated by environmental stress, like heat, osmotic shock and UV light, as well as inflammatory cyokines. It is thought that the pro-inflammatory or cytoprotective response in EC is regulated in part by the balance between the pro- or anti-inflammatory MAPK activities (Hoefen and Berk, 2002) (Figure 1.4).

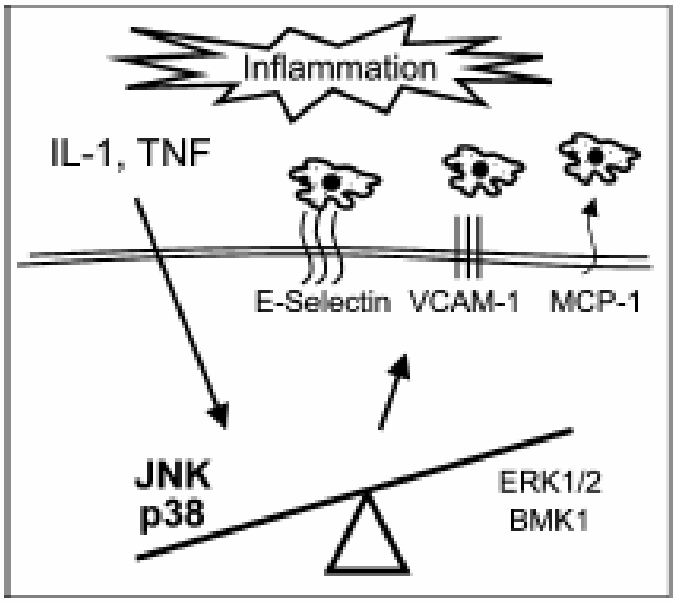


**Figure 1.4 MAPK involvement in endothelial activation and inflammation** The pro-infllammatory MAPKs; p38 and JNK, are activated in response to environmental stress and promotes an inflammation (Hoefen and Berk, 2002)

Activated forms of p38 and JNK are known to further facilitate transcription of pro-inflammatory cytokines (e.g. IL-8, IL-12, MCP-1, and TNFα) and adhesion molecules (e.g. E-selectin and VCAM-1) (Dong *et al.*, 2002). ERK1/2 however, may play a predominantly cytoprotective role in EC by suppressing the activity of JNK or p38

(Hoefen and Berk, 2002) or by upregulating eNOS (Nakata *et al.*, 2005). ERK and BMK1 are as previously mentioned primarily involved in growth and cytoprotective functions (Hoefen and Berk, 2002) and are not considered further in this study.

### 1.3.1. p38

p38 is a member of the MAP kinase proteins and is known to be an important regulator of many diverse processes: inflammation, cell growth and differentiation, and cell survival. In this project I will focus on the pro-inflammatory properties of p38.

p38 has a molecular weight of 38 kDa and there are five isoforms known: p38α, p38β, p38β2, p38δ, p38γ, where p38α (often denoted as just p38) is best characterized and suggested as the most important isoform involved in inflammatory response (Kumar *et al.*, 2003). p38α and p38β are ubiquitously expressed, while the other isoforms are differentially expressed depending on tissue type (Zarubin and Han, 2005).

Under quiescent conditions p38 resides in the cytoplasm in an unactive state. However, in response to cytokines (e.g. TNFα or IL-1), growth factors or stress, p38 is known to be dually phosphorylated on Thr180 and Tyr182 (Herlaar and Brown, 1999). MKK3 and MKK6 are the two main MKKs that are known to activate p38, although MKK4, an upstream kinase of JNK, has been suggested to aid in the p38 activation (Zarubin and Han, 2005).

Further upstream, the MKKs are activated by a diverse range of MKKKs (MAP3Ks), including TAK1, ASK1/MAP3K5, DLK/MUK/ZPK and MEKK4 (Zarubin and Han, 2005). The MAP3Ks are in turn activated by Rho-family proteins, such as Rac and cdc42 (Zarubin and Han, 2005). Many of the p38 MAP3Ks are also involved in activation of the JNK pathway, and this is a possible reason for why these two pathways often are co-activated.

Activated p38 triggers pro-inflammatory signalling by activating transcription factors, and this pathway is strongly linked to diseases like Alzheimers', rhematoid arthritis, inflammatory bowel disease, and possibly atherosclerosis. Through activation of transcription factors such as ATF1/2/6 and Elk-1, p38 plays an important role in the production of pro-inflammatory cytokines (e.g. IL-1, IL-6 and TNFα) and cyclooygenase-2 (COX-2) (Guan *et al.*, 1998), as well as adhesion molecules such as VCAM-1 (Pietersma, 1997).

### 1.3.2. JNK

JNK (sometimes denoted as Stress activated protein kinase, SAPK) is a member of the MAPK family and is involved in pro-inflammatory signalling transduction in response to various stimuli such as cytokines (e.g. TNFα and IL-1) and mechano-sensory stimulation by shear. There are 3 known members of the JNK family: JNK1 (46 kDa), JNK2 (54 kDa) and JNK3.

Cytokines or extracellular stress (e.g. shear stress from fluid flow) will through TLRs or mechanosensory receptors activate Rac or cdc42 which in turn leads to activation of MAP3Ks, including transforming growth factor-β activated kinase 1 (TAK1). The MAP3Ks will futher phosphorylate MKK4 and/or MKK7 which is followed by dual phosphorylation of JNK on its Thr-Pro-Tyr residues. This in turn leads to phosphorylation and nuclear translocation of c-Jun, JunB, JunD and ATF2 (Ip and Davis, 1998) which are members of the activator protein 1 (AP-1) superfamily of transcription factors. AP-1 family members form dimers and are known to transcribe pro-inflammatory genes such as MCP-1.

Recent studies has revealed that an upstream MAP3K, TAK1, is crucial for JNK activation in response to cytokines (e.g. IL-1 and TNF-α) and for JNK activation through TLRs (Weston and Davis, 2007) (Figure 1.5). It is further thought that JNK activation in response to cytokines is mediated thorough MKK7, while MKK4 mediates activation in response to enviromental stress (Davis, 2000)

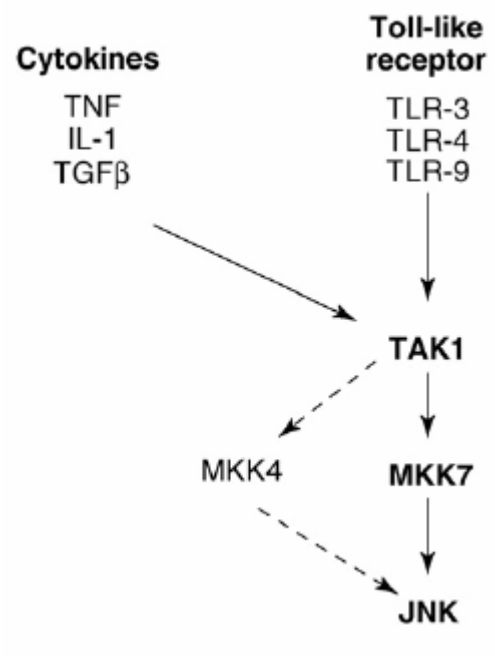


**Figure 1.5 The role of TAK1 in JNK signalling** TAK1 plays a central role in JNK activation mediated by inflammatory cytokines and Toll-like receptors (adapted from Weston and Davis, 2007).

## 1.4. Nuclear factor- κB (NF-κB)

NF-κB is an important transcription factor in pro-inflammatory-, cytoprotective- and anti-apoptotic signalling transduction. It is previously shown that endothelial cells upon activation express high levels of IL-8, MCP-1 and E-selectin among other proinflammatory proteins, and it is commonly thought that this inflammatory response is mainly due to NF-κB (p65) and MAPK signalling.

p65 (RelA) is a subunit in the NF-κB family of proteins along with RelB, c-Rel, NF-κB1 (p50 and its precursor 105) and NF-κB2 (p52 and its precursor p100). NF-κB is a transcription factor that forms either homodimers or heterodimers. NF-κB proteins share a conserved Rel homology domain (RHD) in their N-terminal region that is involved in

DNA binding and dimerization and interaction with the members of the inhibitor of κB (IκB) family. The p65-p50 heterodimer was defined as the classical NF-κB binding form, but this is only one of the multiple species that can bind to κB sites on the DNA strands. The most prevalent activated form of NF-κB is a heterodimer consisting of a p50 subunit and p65. The diverse regulation of NF-κB-dependent promoters is due to the ability of different dimers to bind to distinct κB sites in a cell-type- and stimulus-dependent manner.

Under quiescent conditions the NF-κB dimer is sequestered in the cytoplasm by IκB. The IκB family is composed of IκBα, IκBβ, IκBγ, IκBε, Bcl-3 and the NF-κB precursors p100 and p105 and is characterised by the presence of ankyrin repeats (Whiteside and Israel, 1997). IκBs exert their inhibitory effects via the ankyrin region which binds to the RHD domains of NF-κB dimers. This interaction blocks NF-κB nuclear translocation by masking its nuclear localisation signal (Karin, 1999). However, upon activation by cytokines or stress (e.g. oxidative stress or acute shear stress) IκB is degraded by proteosomes, and NF-κB translocates to the nucleus and commence transcription of pro-inflammatory genes (Figure 1.6).

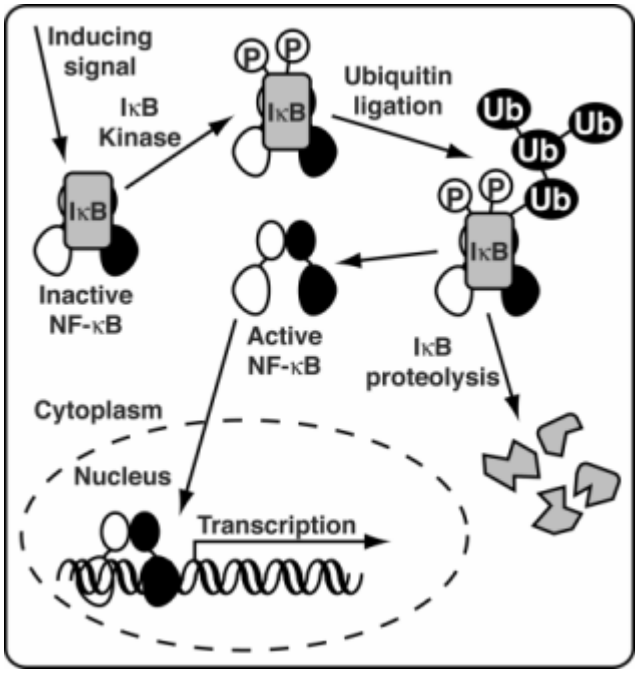


**Figure 1.6 Activation of NF-κB** IκB is phosphorylated, which leads to its ubiquitination. This signals a proteasome to degrade IκB and allows NF-κB to translocate into the nucleus and bind to κB (Internet reference 2)

## 1.5. Resolution of inflammation

The inactivation of inflammation is an active process that operates at different levels. It involves depletion of inflammatory cells by apoptosis and phagocytosis. It also involves inactivation of NF-κB and AP-1 transcription factors thus suppressing expression of pro-inflammatory genes.

### 1.5.1. Downregulation of MAPK activity

MAP kinase activation is often transient, and the level of total MAP kinase does not change throughout the course of the activation – inactivation, thus dephosphorylation is the main mechanism of inactivation (Zarubin and Han, 2005). An important group of

dual-specificity phosphatases are known as the MAP kinase phosphatases (MKPs), where MKP-1 is the most extensively studied.

There is a positive feedback loop in the regulation of p38 and JNK in that they both get activated in response to cytokines (e.g. IL-1 and TNFα) and upon activation they lead to increased expression of these same cytokines. There is however, a downregulation mechanism of MAPK through transcription of MKP-1 (Wadgaonkar *et al.*, 2004), a molecule that dephosphorylates activated MAP-kinases and thereby blocks downstream MAP-kinase signalling transduction (Wadgaonkar *et al.*, 2004). Although downregulation of JNK is poorly understood, recent studies on knockout mice have showed that knocking down MKP-1 and MKP5 increased JNK activity (Weston and Davis, 2007). Thus it is suggested that the MAPK phosphatases are important in resolving inflammatory responses by inhibiting signal transduction caused by p38 and JNK MAP-kinases.

XIAP is a protein that has been proposed as an important mediator in the anti-apoptotic signalling route in the NF-κB pathway by blocking NF-κB crosstalk with JNK and thereby inhibiting pro-inflammatory signal transduction (Nakano, 2004). The upregulation of XIAP may thus be an important gene to investigate further.

Nrf2 is a transcription factor that resides in the cytoplasm bound to Keap-1 under quiescent conditions. Keap-1 is a cytoplasmic protein that is associated with the cytoskeleton. It is known to bind to Nrf2 and thus sequester this transcription factor in the cytoplasm (Mann *et al.*, 2007). Upon activation, Nrf2 dissociates from Keap-1 and

translocates into the nucleus where it binds to antioxidant response element (ARE) promoters and thereby induce transcription of antioxidant genes (Mann *et al.*, 2007). Importantly, Nrf2 is known to suppress the activity of p38 and is thus potentially implicated in the resolution of inflammation.

### 1.5.2. Suppression of NF-κB

Suppression of NF-κB is controlled by multiple negative feedback mechanisms involving NF-κB-dependent induction of multiple regulatory proteins (IκB and A20).

Export of NF-κB from the nucleus is known to terminate transcription of NF-κB dependent genes (Arenzana-Seisdedos et al, 1997 and Rodriguez *et al.*, 1999). This step is carried out by IκBα that is newly synthesised in response to NF-κB activation. This IκBα binds to nuclear NF-κB and shuttles it back to the cytoplasm where it is then retained in an inactive form (Huang *et al.*, 2000).

The A20 gene is activated by NF-κB (Krikos *et al.*, 1992). In addition, A20 suppresses activation of NF-κB and induction of pro-inflammatory transcripts in response to TNFα or IL-1 (Jaattela *et al.*, 1996 and Krikos *et al.*, 1992). The mechanism is not fully understood, but it is thought that A20 acts through binding to tumor-necrosis-factor-receptor-associated-factor (TRAF) molecules which are necessary for pro-inflammatory signalling (Evans P.C., 2001). Elevated levels of A20 will therefore block the pro-inflammatory transciption through the NF-κB signalling pathway in response to stimuli such as cytokines or flow.

Cezanne is a relatively recently discovered molecule in the A20-family. It is a zinc finger protein that is shown to play a part in NF-κB downregulation (Evans P.C., 2001). Thus it is thought to have a cytoprotective role in endothelial cells.

## 1.6. Inflammatory processes in atherosclerosis

Atherosclerosis is regarded as a chronic inflammatory disease (Ross, 1999) and intimal hyperplasia occurs at sites of inflammation. As a graft is inserted into the bloodstream and reperfused, altered hemodynamics will activate the endothelial layer of the graft, and inflammation will commence. Leukocytes and platelets adhere to the inflammation site and macrophages transmigrate into the subendothelial layers. These macrophages accumulate oxidised LDL and differentiate into foam cells. The foam cells produce growth factors that cause the smooth muscle cells (SMC) in the medial layer to proliferate. The SMC start to migrate to the intima and continues their proliferation. The intima is further thickened and underlying fibroblasts start to migrate to the damaged area, differentiating into myofibroblasts (Mitra *et al.*, 2006). At these sites of intimal hyperplasia, atherosclerosis is prone to occur due to altered flow patterns and loss of athero-protective response from prolonged high laminar shear (Sheikh *et al.*, 2002; Sheikh *et al.*, 2005; Yamawaki *et al.*, 2003).

### 1.6.1. Coronary artery bypass grafting

When atherosclerotic lesions form in the coronary arteries, the lesion might eventually lead to ischemic heart disease, followed by myocardial infaction. In some cases, patients

are treated by re-opening the occluded vessel by balloon angioplasty or stenting. However, in many cases the patient needs surgical revascularisation (or coronary artery bypass grafting), in which an endogenous artery or vein is transposed into the blocked coronary artery "below" the point of occlusion, and thereby supplies the mycardium with freshly oxygenised blood (Figure 1.7). Indications for coronary artery bypass grafting (or simply bypass surgery) are most often angina that persists or progress despite medical therapy and prolongation of life in high-risk patients (Canver, 1995).

The conduit to be used during coronary artery bypass grafting depends, in part, on the anatomical site of the occlusion and the clinical condition of the patient. The internal mammary artery (or the internal thoracic artery) is, among cardiothoracic surgeons, the preferred conduit (Gardner, 2007). However, in patients with multiple occlutions or when the internal mammary artery is unsuited as a conduit other options has to be considered, and the greater saphenous veins is often the conduit of choice.

Human saphenous vein grafts are the most used conduits in cardiac surgery (Canver, 1995), however their use is usually complicated by high rates of late stenosis and eventual luminal occlusion due to the development of intimal hyperplasia and accelerated atherosclerosis. Outside of early graft thrombosis which is frequently technically related, vein grafts can develop accelerated intimal hyperplasia as early as one month following surgery (Mitra *et al.*, 2006). Accelerated intimal hyperplasia can significantly reduce lumen area, and increases the propensity of veins to develop accelerated atherosclerosis causing failure of up to 40% of vein grafts within 10 years of surgery (Motwani and

Topol, 1998), leading in turn to recurrence of clinical symptoms such as angina, and increasing the risk of re-infarction. It is therefore interesting to note that, the internal mammary artery grafts, when compared to venous grafts, are almost resistant to restenosis (Mitra *et al.*, 2006).

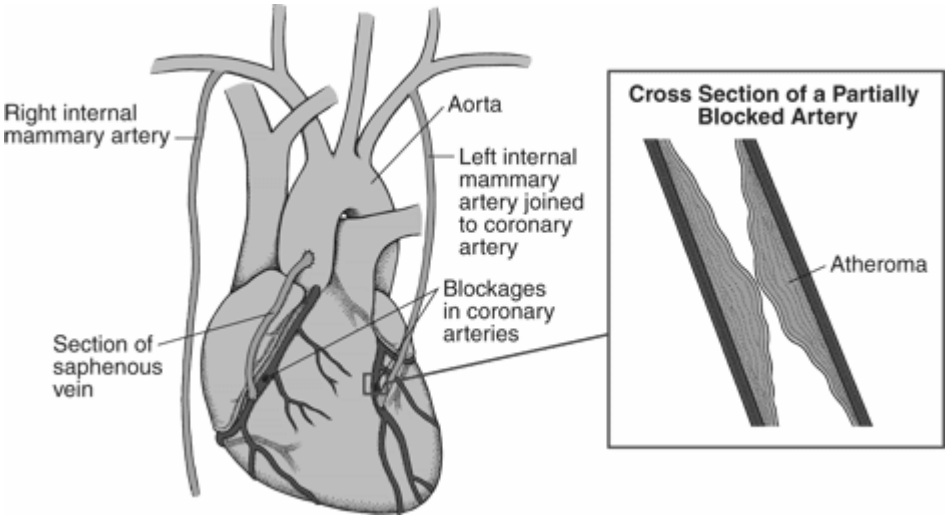


**Figure 1.7 Bypass surgery using either a saphenous vein graft or a internal mammary artery** Coronary artery bypass surgery consists of attaching an artery or part of a vein to a coronary artery, so that the blood has an alternate route to the coronary arteries. As a result, the narrowed or blocked area is bypassed (Internet reference 3)

### 1.6.2. Monocyte-macrophages regulate accelerated atherosclerosis in vein grafts

Vein grafts are particularly susceptible to accelerated atherosclerosis which is characterised by the influx of inflammatory cells and proliferation of smooth muscle cells, processes that lead to thickening of the intima.

Several lines of evidence suggest that inflammatory cells contribute to the pathogenesis of accelerated atherosclerosis in vein grafts: Firstly, macrophages have been detected at early stages following vein to artery transposition in humans (Amano *et al.*, 1991) and rats

(Hoch *et al.* 1994) and macrophages are more abundant in vein grafts than in native atherosclerotic lesions (Ratliff and Myles, 1989). Secondly, the process of vein grafting induces expression of adhesion molecules (Kwei *et al.*, 2004) and chemokines (Stark *et al.*, 1997) in EC within 6-24 hours following surgery, thus facilitating early inflammatory responses. Finally, vein graft disease is reduced by depletion of macrophages (Hoch *et al.*, 1999) or by genetic deletion of known pro-inflammatory genes such as ICAM-1 (Zou *et al.*, 2000) or the p55TNF receptor (Zhang *et al.*, 2004), which suggests that inflammatory cells contribute to pathogenesis. Thus prevention of early inflammation may protect from intimal hyperplasia and secondary atherosclerosis in vein grafts.

### 1.6.3. Inflammation-related signalling mechanisms in vein graft endothelium

Pro-inflammatory activation of EC requires the activation of MAPK and NF-κB signalling pathways (Ghosh *et al.*, 1998 and Dong *et al.*, 2002 ) which makes them potential targets for anti-inflammatory therapies.

Pro-inflammatory signalling generates active, phosphorylated forms of JNK and p38 (Dong *et al.*, 2002) which activate transcription factors belonging to the activator protein-1 (AP-1) family (including c-Jun, ATF2 and c-Fos) and other cellular proteins through ATP-dependent phosphorylation (Dong *et al.*, 2002). Studies using cultured cells revealed that the JNK/p38-AP1 signalling pathway is essential for the induction of VCAM-1 (Ahmad *et al.*, 1998), E-selectin (Read and Whitley, 1997 and Min and Prober, 1997) and IL-8 (Natarajan *et al.*, 2001 and Parhar *et al.*, 2003) proteins in activated EC. JNK and p38 are activated rapidly by phosphorylation in vein bypass grafts (Saunders *et al.*, 2004

and Cornelissen *et al.*, 2004). However, the exact role of MAP kinases in relation to vein graft disease and their mode of regulation remain largely unknown.

## 1.7. Effects of shear stress on vascular inflammation: potential relevance in vein grafting

Blood flow exerts shear stress (mechanical drag) on vascular endothelium, which varies in time, magnitude and direction according to vascular pulsatility and anatomy. Artery walls are exposed to much higher shear stresses (12-20 dynes/cm2) compared to vein walls (<5 dynes/cm2). It is widely believed that shear stress regulates the susceptibility of the arterial tree to atherosclerosis by altering EC physiology. Prolonged high laminar shear suppresses atherosclerosis in regions of the arterial tree with uniform geometry by exerting several 'protective' effects on EC, including inhibition of the cell cycle (Lin *et al.*, 2000), suppression of thrombosis (Grabowski *et al.*, 2001), promotion of viability (Dimmeler *et al.*, 1996) and suppression of inflammatory activation (Sheikh *et al.*, 2003; Sheikh *et al.*, 2005; Yamawaki *et al.*, 2003). In contrast, atherosclerosis occurs predominantly at arches and branches of the arterial tree that are exposed to low shear or complex flow patterns (Dai *et al.*, 2004 and Cunningham and Gotlieb, 2005).

Shear stress regulates vascular physiology by altering EC through mechanosensory receptors which converts mechanical forces into biochemical signals (Grabowski *et al.*, 2001; Lan et al, 1994; Lin *et al.*, 2000; Sheikh *et al.*, 2003; Yamawaki *et al.*, 2003). Exposure of cultured EC to high laminar shear has profound effects on pro-inflammatory signalling. *Acute* induction of arterial shear stress (5m - 4h) is known to activate JNK (Li

*et al.*, 1996 and Miao *et al.*, 2002) and NF-κB (Bhullar *et al.*, 1998; Davis ME *et al.*, 2004; Hay *et al.*, 2003; Lan *et al.*, 1994) and trigger the expression of ICAM-1 (Burns and DePaola, 2005; Morigi *et al.*, 1994a; Nagel *et al.*, 1994), VCAM-1 (Gonzales and Wick, 1996) and E-selectin (Burns and DePaola, 2005) in EC, thus facilitating leukocyte adhesion (Burns and DePaola, 2005; Gonzales and Wick, 1995; Morigi *et al.*, 1995; Nagel *et al.*, 1995). Exposure of venous endothelial cells to arterial flow following transposition to the arterial circulation could thereby lead to activation of pro-inflammatory signaling pathways and expression of adhesion proteins, chemokines and cytokines.

## 1.8. Hypothesis and aims of present study

We hypothesise that the relative susceptibility of vein grafts to inflammation compared to artery grafts may be a consequence of different responses of venous EC and arterial EC to shear stress induced by arterial blood flow.

We now wish to know:

(i) whether elevating shear from venous (<5 dynes/cm2) to arterial (12-20 dynes/cm2) levels (or from static to arterial shear) activates cultured porcine jugular vein EC (PJVEC)

(ii) whether the induction of pro-inflammatory mRNA is accompanied by indiction of cytoprotective transcripts (MKP-1, A20, Cezanne, XIAP, Keap-1, Nrf2)

(iii) whether PJVEC respond to flow in the same way as aortic EC

(iv) whether inactivation of MAP kinases can suppress induction of pro-inflammatory molecules by arterial shear in PJVEC

# 2. MATERIALS AND METHODS

## 2.1. Isolation and culturing of endothelial cells

### 2.1.1. PAEC isolation

Porcine aortas was obtained from a local abattoir and kept in transport medium consisting of Hanks' balanced salt solution (HBSS), 0.5% penicillin-streptomycin, 0.5% gentamicin and 0.5 % sodium-pyruvat. To isolate the PAEC the aortas were first dissected and cleansed in a sterile laminar air flow hood to get rid of the surrounding adipose tissue. One end of the aorta was clamped shut using one or more surgical scissor clamps and all the branches were tied off using surgical thread. The aorta was filled with collagenase (0.5mg/ml) in HBSS (40 ml) and clamped shut at both ends. The aorta was covered in foil and incubated for 15 minutes at 37 ˚C. The collagenase was flushed through and put into a 50 ml tube. The aorta was washed once with 10ml HBSS w/o and this wash was added to the tube. The tube was spun at 1200 RPM and the supernatant was discarded. The pellet was resuspended in another 50 ml of HBSS and spun once more at 1200 RPM. This time the pellet was resuspended in 5 ml complete M199 media (Sigma). The cell suspension was transferred into at T25 flask coated with 1% gelatin and incubated at 37 ˚C. After approximately 24 hours the medium was changed to remove dead cells and other debris. After approximately 2-3 days the T25 flask was confluent and ready for splitting.

### 2.1.2. PJVEC isolation

Porcine jugular veins were obtained from a local abattoir and the cell harvesting from the veins was done in the same manner as with the PAEC, although the veins are far more fragile and has to be handled more gently than the aortas. After approximately 24 hours the medium was changed to remove dead cells and other debris. In the following weeks the medium was changed on the cells every 2-3 days. To get a confluent T25 flask took approximately 6 weeks of gentle care.

We experienced some complications in culturing the PJVEC and we lost several of our cultures. This was mainly due to infections. In addition, incidents of foot and mouth disease in the UK made it difficult to obtain new porcine tissue at times from which we could harvest more cells. This, and the fact that it took approximately 6 weeks for the cells to reach confluency, made it difficult to get enough cells for our experiments during this project.

### 2.1.3. Complete medium for porcine EC

Complete medium for porcine EC was made by adding 100 ml heat inactivated fetal calf serum (FCS, Sigma), 5 ml L-glutamin (200 mM, Sigma) and 5ml penicillin-streptomycin (10,000 units/ml penicillin and 10 mg/ml streptomycin (Sigma)) to a 500 ml bottle of medium M199 (Sigma). Immediately prior to use, 10 µl/ml of ECGF (Endothelial cell growth factor from bovine neural tissue (Sigma) dissolved in 1000 IE/ml Heparin (Monoparin)) was added to complete the medium.

### 2.1.4. Passaging PAEC

After the cells reached 80 - 90 % confluency, usually after 2-3 days, the cells were split 1:3. The medium was removed by a vacuum suction pump, and the cells were briefly rinsed with HBSS w/o. Trypsin-EDTA (2 ml in a T25 flask, 5 ml in a T75 flask) was added to the flasks and they were incubated for about 2-3 minutes at 37 ˚C. Trypsin is a serine protease which is used to resuspend the cells in cell-culture flasks. It acts by hydrolysing the peptides that adheres the cells to the gelatin base in the flask. After the incubation with trypsin the flasks were tapped on the side to lift the cells into suspension. The suspension was then transferred into a 50 ml tube. The flask was washed once with HBSS w/o and this wash was added to the tube. The tube was filled ad 50 ml with HBSS w/o and spun down at 1200 RPM. The supernatant was discarded and the pellet resuspended in HBSS w/o and spun once more at 1200 RPM. The supernatant was once more discarded and this time the cell pellet was resuspended in complete media (5 ml per flask). The cell suspension was homogenized by pipetting with a 10 ml pipette, and then divided into fresh flasks coated with 1 % gelatin.

### 2.1.5. Passaging PJVEC

After the cells reached 80 - 90 % confluency, usually after approximately 6 weeks for the initial T25 flask, the cells were split 1:3. However, after the first passage the cells grew faster and usually reached 80-90 % confluency in 4-5 days. The medium was removed by a vacuum suction pump, and the cells were briefly rinsed with HBSS. Trypsin-EDTA (2 ml in a T25, 5 ml in a T75 flask) was added to the flasks and they were incubated for about 2-3 minutes at 37 ˚C. After the incubation with trypsin the flasks were tapped on

the side to lift the cells into suspension. The suspension was then transferred into a 50 ml tube. The flask was washed once with HBSS and this wash was added to the tube. The tube was filled ad 50 ml with HBSS and spun down at 1200 RPM. The supernatant was discarded and the pellet resuspended in HBSS and spun once more at 1200 RPM. The supernatant was once more discarded and this time the cell pellet was resuspended in complete media (5 ml per flask). The cell suspension was homogenized by pipetting up and down with a 10 ml pipette, and then divided into fresh flasks coated with 1 % gelatin.

## 2.2. Cytodyne parallel-plate flow chamber loop

To mimic the effects of arterial shear we used a Cytodyne parallel-plate flow chamber loop (as shown in Figure 2.1). The apparatus consist of two reservoirs, situated one above the other and with a flow chamber (Figure 2.2) positioned in between them. Medium is pumped into the upper reservoir and flow is driven though the chamber by the hydrostatic pressure head created by the vertical distance between the reservoirs (Frangos *et al.*, 1987). The parts of the apparatus was cleaned and perfused with water before it was autoclaved. The apparatus was assembled in a laminar flow hood and placed in a workbench heated to 37 ˚C and connected to a gassing system that maintained a 5 % $CO_2$, 95 % air atmosphere. The medium used in the experiments was M199 (Sigma), 0.5 % L-glutamin (20mM, Sigma), 0.5 % penicillin-streptomycin(10,000 units/ml penicillin and 10 mg/ml streptomycin (Sigma)).

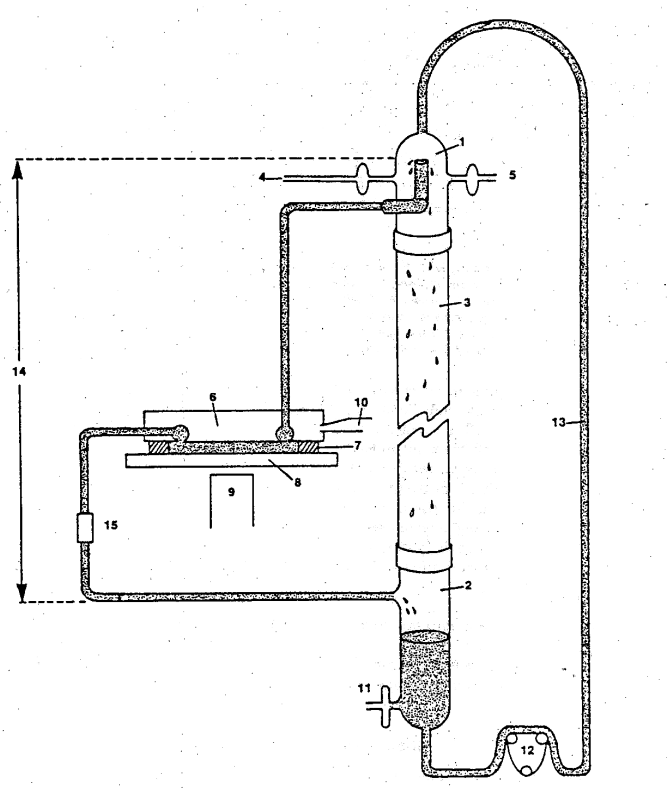


**Figure 2.1 Drawing of small volume flow loop**. (1) upper reservoir, (2) lower reservoir, (3) overflow manifold, (4) filtered humidified 95% air + 5% C02 input, (5) gas outlet, (6) flow chamber, (7) gasket, (8) slide with cell monolayer, (9) microscope objective, (10) vacuum, (11) sampling port, 12) roller pump, (13) PFA teflon tubing, (14) constant pressure head, and (15) flow probe (Frangos *et al.*, 1987).

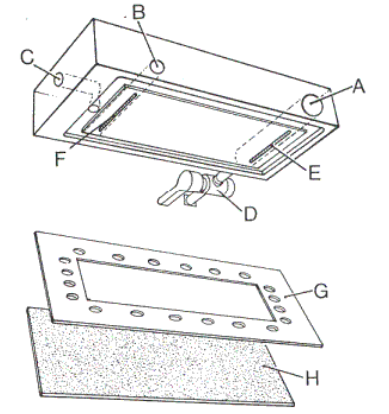


**Figure 2.2 Parallel plate flow chamber**. The polycarbonate plate, the gasket (G), and the glass slide (H) with the attached cells are held together by a vacuum (C), forming a channel of parallel plate geometry. Medium enters at entry port (A), through slit (E), into the channel, and exits through slit (F), and exit port (B). Entry port (A) also serves as a trap for bubbles, which can be removed through valve (D) (Frangos *et al.*, 1987)

Porcine EC were cultured on gelatin coated glass slides for 48 hours before they were mounted in the parallel-plate flow chamber. In our experiments we exposed the cells to 12 dynes/$cm_2$ shear stress, which is equivalent to arterial shear. We exposed the cells to flow for 0, 30, 90, 120 or 240 minutes.

## 2.3. Lysing of EC and protein extraction

Protein lysates were obtained from cells by using the Nucbuster protein extraction kit (Novagen) and the following protocol: The cells were gently rinsed twice with ice cold PBS (phosphate buffered saline (Gibco)) and the PBS was removed by a vacuum suction pump. 150 µl of the lysis buffer (NucBuster Reagent 1) was added to the monolayer of cells and the slide was incubated on ice for 5 minutes. After incubation the cells were scraped off the slide using a cell scraper and transferred into a 1,5ml eppendorf tube. The suspension was vortexed for 15 seconds and incubated on ice for another 5 minutes. This was repeated twice before the suspension was spun down at 16 000 RPM at 4 ˚C. The supernatant now contained the cytoplasmic proteins, and was transferred into a fresh eppendorf tube and stored at –80 ˚C. The pellet was used to make nuclear protein lysates as follows; First it was resuspended in 1 µl protease inhibitor cocktail (supplied with the NucBuster kit), 1 µl of DTT (supplied with NucBuster kit) and 75 µl of NucBuster Reagent 2. It was then vortexed for 15 seconds and incubated on ice for 5 min. This was repeated twice before the suspension was spun down at 16 000 RPM for 10 minutes at 4 ˚C. The supernatant now contained the nuclear protein extract. It was transferred into a fresh eppendorf tube and stored in the –80 ˚C freezer.

### 2.3.1. Protein quantification using BioRad DC protein assay kit

The assay is based on a colorimetric reaction between the protein and an alkaline copper tartrate solution and Folin reagent. The colorimetric reaction consist of two steps: first the protein and copper will form a complex in alkaline solution, which in turn reduces the folin reagent producing a blue colour (BioRad homepage).

The bovine serum albumin (BSA) stock solution is 300 mg/ml in PBS. We diluted the BSA into two working solutions at 10 mg/ml and 1 mg/ml before we made a standard curve according to the following table:

**Table 2.1 BSA protein concentration standard curve**

| | | Volume needed (µl) | | | |
|---|---|---|---|---|---|
| Sample | **BSA concentration (mg/ml)** | Standard dilution buffer (PBS) | BSA 1 mg/ml | BSA 10 mg/ml | Total volume (µl) |
| 1 | **0.0** | 12.0 | 0.0 | 0.0 | 12.0 |
| 2 | **0.1** | 10.8 | 1.2 | 0.0 | 12.0 |
| 3 | **0.2** | 9.6 | 2.4 | 0.0 | 12.0 |
| 4 | **0.4** | 7.2 | 4.8 | 0.0 | 12.0 |
| 5 | **0.6** | 4.8 | 7.2 | 0.0 | 12.0 |
| 6 | **0.8** | 2.4 | 9.6 | 0.0 | 12.0 |
| 7 | **1.0** | 0.0 | 12.0 | 0.0 | 12.0 |
| 8 | **1.3** | 14.0 | 0.0 | 2.0 | 16.0 |
| 9 | **1.5** | 17.0 | 0.0 | 3.0 | 20.0 |

The BioRad protein assay kit contains 3 reagents: A, B and S. We made a solution A' which consist of 20 µl Reagent S in 1 ml of Reagent A. Using a 96 well plate we added to each well 5 µl sample/standard, 25 µl solution A' and 200 µl Reagent B. Each sample or standard were ran in duplicates. We read the plate in a UV spectrophotometer at 750 nm

within 15 minutes. Sometimes the samples were oversaturated and had to be diluted to fall within the concentrations covered by the standard curve.

The readout from the spectrophotometer stated the samples' optical density (OD) values. The values from the BSA standard samples were used to calculate a standard curve in a Microsoft Excel work sheet by using the linear equation $y = ax + b$, where y is the OD (absorbance) and x is the concentration. The values for *a* and *b* are constants which are given when the standard curve is drawn in the work sheet. Since the only unknown is the concentration the final equation is this:

$$(\text{dilution factor}) * \text{concentration} = (OD-b)/a$$

This is the equation used to calculate the samples protein concentration.

## 2.4. Protein expression analysis by western blotting

### 2.4.1. SDS Page gel electrophoresis

SDS Page electrophoresis allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acryl amide mesh of the gel. Smaller proteins migrate faster than larger proteins and the proteins are thus separated according to size.

15 µl of each sample was transferred into a 1.5 ml eppendorf tube. Loading buffer was prepared by having 180 µl NuPage LDS 4X Loading Buffer (Invitrogen) into an eppendorf and adding 20 µl DTT 1M. 5 µl of this mix was added to each sample and the

tubes were put on a heating block set to 95 ˚C for 5 minutes to denature the proteins. The tubes were then spun down for 30 seconds to retrieve the condensation.

A gel (NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm, 12 wells (Invitrogen)) placed in a electrophoresis tank filled with running buffer (40 ml of NuPage Mops SDS Running buffer 20 X added to 760 ml sterile water) was loaded with the sample-loading buffer mix (20 µl per well). One well was loaded with 10 µl marker (SeeBlue Plus2® Prestained standard (1x) (Invitrogen)). The electrophoresis was run for 1 hour at 200 V.

### 2.4.2. Transfer

Transfer was performed using the NuPAGE™ System (Invitrogen). Transfer buffer was made, consisting of 850 ml ultrapure water, 50 ml transfer buffer (NuPAGE™ Transfer Buffer (20x) (Invitrogen)), 100ml absolute methanol and 1 ml antioxidant (NuPAGE™ Antioxidant (Invitrogen)). A PVDF membrane (Immobilon-P Transfer membrane, filter type: PVDF, pore size: 0.45 µm (Millipore)) was wetted for 30 seconds in methanol (Methanol Microscopy (BDH)) and then washed quickly in the transfer buffer before use. Transfer was completed by assembling the gel, membrane, filter paper (Chromatography paper, 3mm, CHR, 58x68 cm (Whatman®) (soaked in transfer buffer) and blotting pads (soaked in transfer buffer). The transfer was run for 1 hour at 25 V.

### 2.4.3. Blocking and protein detection

Blocking of non-specific protein binding was achieved by placing the membrane with the protein side facing up in a dilute solution of protein (Marvel dried skimmed milk, 1% fat) in a 0.1% PBS-Tween solution (1 ml Tween®20 Sigma Ultra (Sigma) in 1000 ml PBS (Gibco)). The membrane was left on blocking on a shaker at room temperature for 1 hour. After the blocking, the membrane was washed in 0.1 % PBS-Tween 3 times for 5 minutes on a rocking platform.

The membrane was then incubated with the primary antibody over night at 4 ˚C on a rocking platform (for primary antibody concentrations, see table 2.2) followed by 3 washes of 5 minutes with PBS-Tween.

**Table 2.2 Primary antibodies used in western blotting (diluted in PBS-Tween w/1% skimmed milk)**

| Company | Primary antibody | dilution factor |
|---|---|---|
| New England Biolabs | mouse anti-phospho p38 | 1:1000 |
| New England Biolabs | rabbit anti-p38 | 1:1000 |
| New England Biolabs | mouse anti-phospho JNK | 1:1000 |
| New England Biolabs | rabbit anti-JNK | 1:1000 |
| Santa Cruz biotechnologies | rabbit anti-p65 | 1:1000 |
| Santa Cruz biotechnologies | goat anti-lamin B | 1:1000 |

The secondary antibody (horse radish peroxidase (HRP) - conjugated) was added to the membrane and left on for 45 minutes on a rocking platform (table 2.3). The membrane was then washed 3 times 5 minutes to get rid of excess antibody.

**Table 2.3 Secondary HRP-conjugated antibodies used in western blotting (diluted in PBS-Tween)**

| Company | Secondary antibody | dilution factor |
|---|---|---|
| Dako | Polyclonal goat anti rabbit | 1:10 000 |
| Dako | Polyclonal rabbit anti mouse | 1:10 000 |
| Dako | Polyclonal mouse anti goat | 1:10 000 |

3 ml of each of the two Western Lightning Chemoluminescence reagents (PerkinElmer) was added to the membrane for 2 minutes to enhance the luminescence. The membrane was quickly dried between two sheets of filter paper and covered in saran wrap foil.

The membrane was brought into the dark room and placed in a light sealed cassette with photographic film (KODAK®BioMax Light Film 18x24 cm Light-1 (Sigma-Aldrich)) on top and left for appropriate times of exposure. After exposing the film to the luminescence, the film was developed using an automated developer (Compact X4 Automatic X-ray Film Processor (Xograph Imaging Systems)).

### 2.4.4. Analysing western blots by densitometry

We analyzed the western blots by densitometry, using the Image-J software. The levels of expression of the proteins of interest were given by calculating the ratio of the target protein and an internal standard. Levels of active, phosphorylated forms of p38 or JNK in the cytoplasm, were normalised by measuring the amount of total p38 or JNK in the cytoplasm. Similarly, levels of p65 in the nucleus were normalised by measuring the amount of lamin B (a nuclear protein) in the nucleus.

## 2.5. RNA isolation

The RNA was extracted using the RNeasy Mini kit (Qiagen). Untreated, flow treated and TNFα treated cells were rinsed briefly with ice cold PBS. The PBS was completely aspirated and 350 µl of the denaturing Buffer RLT + BME (β-mercaptoethanol) (10 µl BME per 1ml of Buffer RLT) was added to inactivate RNases and to ensure a high yield of intact RNA. The cells were disrupted and collected by using a cell scraper and the suspension was transferred into a 1.5 ml eppendorf. The sample was homogenized by using a syringe and needle (20 gauge, 0.9 mm) and pipetting the suspension up and down 10 times. Homogenization is important to reduce the viscosity of the suspension and to avoid flocculation of RNA. Incomplete homogenization gives significantly reduced yield and may lead to clogging of the RNeasy Mini Spin Column.

To the lysed and homogenized cells, 350 µl of 70 % ethanol was added and mixed gently using a pipette. This mix was added into a RNeasy spin column placed in a 2 ml collection tube and spun down at 10 000 RPM fro 15 seconds. The flow through was discarded. To wash the sample 700 µl of RW1 buffer (supplied with kit) was added to the column and it was spun down at 10 000 RPM for 15 seconds. The flow through and collection tube was discarded. The RNeasy spin column was placed in a new 2 ml collection tube, and 500 µl of diluted RPE buffer was added to the column to further wash the sample. Then it was spun down at 10 000 RPM for 15 seconds. The flow through was discarded and another 500 µl of RPE-ethanol was added to the column. It was spun down at 10 000 RPM for 2 minutes to dry the column and thereby avoid ethanol carryover that would contaminate the sample. The collection tube and flow through was

discarded and the column was placed in a 1.5 ml collection tube. 40 µl of Rnase-free water was added directly to the spin column silica-gel membrane and it was spun down at 10 000 RPM for 1 minute to elute the pure RNA. The RNA was stored at – 80 ˚C.

### 2.5.1. Quantification of RNA using UV spectrophotometer

The spectrophotometer (Ultrospec®3000 UV/Visible Spectrophotometer (Pharmacia Biotech)) was programmed to read the optical absorbance at 260 nm and 280 nm. The approximate RNA concentration can be calculated by the ratio between the two measurements at 260 and 280 nm. The quartz cuvette was washed with ultra pure water and dried with a paper towel. The RNA samples were diluted 1:100 in RNase-free water and mixed well. A sample of 100 µl ultra pure water was used as a blank sample to calibrate the spectrophotometer. 100 µl of each sample was in turn added to the cuvette and analysed, the cuvette was rinsed with 100 µl of ultra pure water between each sample.

### 2.5.2. Complementary DNA (cDNA)

cDNA was synthesised from mRNA using the reverse transcriptase enzyme. Given the RNA concentration in each sample we calculated the amount needed for 1 µg of RNA and adjusted the volume to 15 µl with ultra pure water. I then added 1 µl oligo dT and 1 µl dNTP to each sample. Then the tubes were put on a heating block set to 65 ˚C for 5 minutes and were put directly onto a tray of ice to cool down after heating. To each tube 5 µl 5x First strand buffer, 2 µl DTT and 1 µl SuperScript II was added. The tubes were

put onto a heating block set to 42 ˚C for 45 minutes then rising to 70 ˚C for 15 minutes to make the finished cDNA.

## 2.6. Quantitative real-time polymerase chain reaction (PCR)

### 2.6.1. Real time RT-PCR principle

The real time PCR is an effective method for quantifying levels of mRNA expression. It is based on repeating cycles of 3 stages (Figure 2.3): denaturation, annealing and polymerisation/elongation. In the denaturation step the reaction is heated to 94-98 ˚C to heat inactivate most enzymes (but not the heat stable iTaq DNA polymerase) and to denature the DNA into single strands. After the denaturation step the reaction is quickly cooled to prevent reformation of the DNA strands. Yet, because of their small size the primers anneal to their complementary single strands. After annealing the temperature is raised to the polymerisation optimum temperature, which for iTaq polymerase is 70-74 ˚C. During each cycle the transcripts will theoretically be amplified 2 fold, and by repeating the cycle over and over the amplification will be logarithmic, where the amount of transcript will be $2^n$, where n is the number of cycles. Eventually the reaction will reach a plateau phase and level out. There is a linear relationship between the amount of DNA and the cycle number.

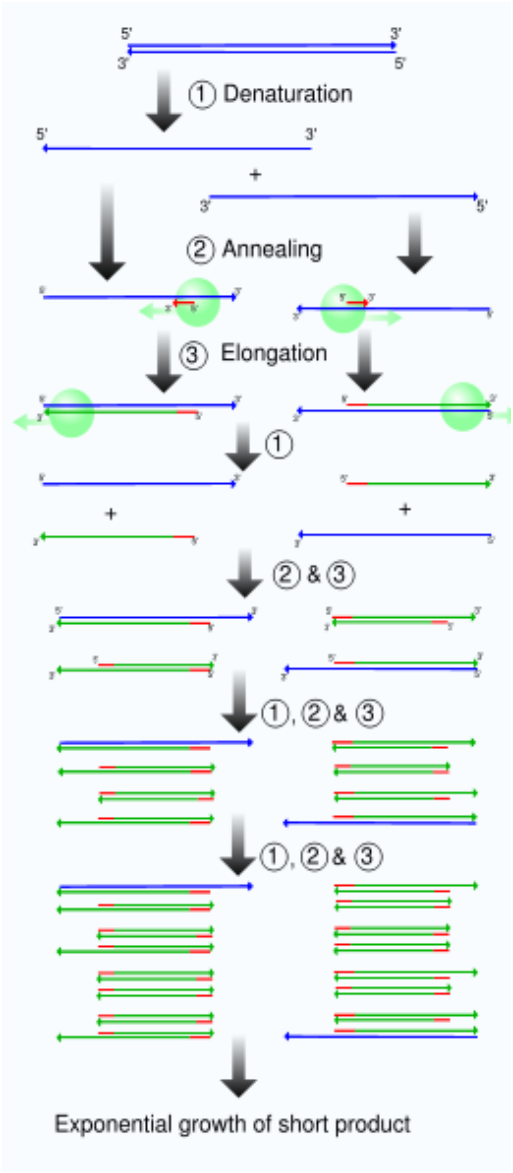


**Figure 2.3 The PCR cycle.** Diagram of PCR reaction to demonstrate how amplification leads to the exponential growth of a short product flanked by the primers. Schematic drawing of the PCR cycle. 1: Denaturing at 96°C. 2: Annealing at 68°C. 3: Elongation at 72°C (P=Polymerase). The first cycle is complete. The two resulting DNA strands make up the template DNA for the next cycle, thus doubling the amount of DNA duplicated for each new cycle (Internet reference 4)

### 2.6.2. RT-PCR plate setup

cDNA was diluted 1:10 with ultra pure water to working concentration. The primers stock concentration was 100 µM and they were diluted 1:10 with ultra pure water to the working concentration 10 µM. For each gene we made up a master mix with the following constituents and amounts per well:

12.5 µl Sybr Green Jumpstart Readymix
6.5 µl ultra pure water
0.5 µl forward primer
0.5 µl reverse primer

The reaction was set up in an optical reaction 96 well plate (BioRad), and samples were run in triplicates for each gene. To each well 5 µl of working concentration sample (cDNA) and 20 µl of the mastermix was added. When the pippeting was complete the plate was covered with optical adhesive film and the plate was vortexed for 15 seconds before it was spun down at 1000 RPM for 1 minute. The plate was then mounted into the BioRad iCycler ®.

### 2.6.3. Primer design

The primers define which fragments of DNA that are to be amplified and are thus of crucial importance to get a successful result when using real time PCR. The primers are short strands of nucleic acids that are perfect complements to the starting point of the gene sequence that is to be amplified. Since DNA polymerases (iTaq) can only bind to double stranded DNA the primers serve as a starting point for replication of the DNA

fragment in question. Primers for porcine versions of several pro-inflammatory and anti-inflammatory genes were designed (table 2.4).

**Table 2.4 Primer sequences used in PCR**

| Gene | Forward sequence | Reverse sequence |
|---|---|---|
| Cyclophilin | 5' ATTTGATGATGAGAATTTTATCC -3' | 5'-ATGCCCTCTTTCACTTTG-3' |
| MCP-1 | 5'-GTCACCTGCTGCTATACACTTAC-3' | 5'-ATCACTGCTTCTTTAGGACACTTG-3' |
| IL-8 | 5'-CACCATGACTTCCAAACTGGC-3' | 5'-CTGCTGTTGTTGTTGCTTCTC-3' |
| VCAM-1 | 5'-GTGTGCGAGGGAGTTAATC-3' | 5'-CACTAGAGCAGGTCATGTTC-3' |
| ICAM-1 | 5'-ATCAATGGAACCGAGAAG-3' | 5'-CACTCTATGCTCACTGTAG-3' |
| E-selectin | 5'-TTCAAATCCTCCCTTACAC-3' | 5'-CATAGAGACCATCAATAGC-3' |
| P-selectin | 5'-TGAGCCTTGTGTGAAAAG-3' | 5'-GTCCATAGAATCCAGAGTAG-3' |
| IP-10 | 5'-CCTGTTAATCCGAGGTCCTTAGAAAA-3' | 5'-ATTGTGGCAATGATCTCAACATGT-3' |
| MKP-1 | 5'-GCAAACATACAACTGTTGGCA-3' | 5'-TAGATCCTGAAGCAGGTTTGTTT-3' |
| XIAP | 5'-GAGGAGGGCTAACAGATTGG-3' | 5'-GATACCACTTCGCATGCTGT-3' |
| Keap-1 | 5'-CCTCATCGAGTTCGCTTACA-3' | 5'-CATTCATGACATGGAGCACA-3' |
| Nrf2 | 5'-AGCAAGTTTGGGAGGAGCTA-3' | 5'-GTCGGTTTGGTTTCTGGACT-3' |
| A20 | 5'-AAACGAAATTCCATGCAACA-3' | 5'-GCTTCTCCCAGGTCTGGTC-3' |
| Cezanne | 5'-ACAATGTCCGATTGGCCAGT-3' | 5'-ACAGTGGGATCCACTTCACATTC-3' |

### 2.6.4. Detection of products

In real time PCR the amount of product is assessed at the end of each cycle. This requires incorporation of fluorescent labeling, and we used SYBR Green for our detection. SYBR Green is a DNA binding dye which exhibits little or no fluorescence while unbound. However, during the annealing and elongation steps the dye molecules bind to the double stranded DNA and emit light upon excitation (Figure 2.4). In real time PCR, the fluorescence is measured at the end of the elongation step in each cycle to monitor the increasing amount of DNA.

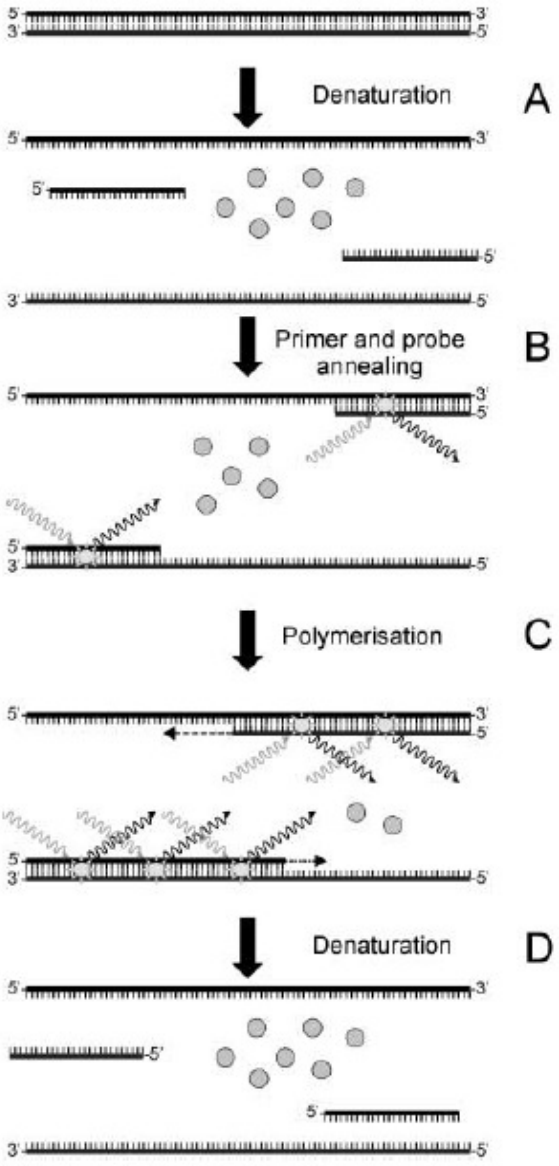


**Figure 2.4 Dye incorporation method.** (A) During denaturation, unbound SYBR Green I dye exhibits little fluorescence. (B) At the annealing temperature, a few dye molecules bind to the double-stranded primer/target, resulting in light emission upon excitation. (C) During the polymerization step, more and more dye molecules bind to the newly synthesized DNA, and the increase in fluorescence can be monitored in real-time. (D) On denaturation, the dye molecules are released and the fluorescence signal returns to background (Bustin, 2000)

### 2.6.5. Calculation of transcript levels

The fluorescence values are recorded at the end of every cycle and represent the amount of product amplified to that point in the amplification reaction. The more template cDNA present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background and this point is defined as the cycle threshold (Ct) (Gibson *et al.* 1996).

Cyclophilin is a transcript that is constitutively expressed in porcine EC and is commonly used as a housekeeping gene in PCR experiments on porcine cells (Dozois et al, 1997). Cyclophilin was used as the internal standard to which the samples were normalized.

$$\Delta Ct = Ct \text{ (target gene)} - Ct \text{ (housekeeping gene)}$$

The comparative Ct ($\Delta\Delta Ct$) method was used to quantify the relative changes in mRNA expression in treated samples compared to a untreated control sample (baseline).

$$\Delta\Delta Ct = \Delta Ct \text{ (sample)} - \Delta Ct \text{ (control)}$$

Then the $\Delta\Delta Ct$ values were transformed to absolute values:

$$\text{Comparative transcription level} = 2^{-\Delta\Delta Ct}$$

## 2.7. NF-κB p65 ELISA kit (TransAm)

The kit contains strips of wells coated with an immobilized oligonucleotide that is recognized by p65. p65 in the sample will specifically bind to the oligonucleotide and by using an antibody that is directed against p65 the NF-κB dimer is detected. Addition of a HRP-conjugated secondary antibody makes quantification by spectrophotometry possible.

Nuclear protein lysates from PAEC treated with 0, 30 or 90 minutes of flow were tested in duplicates with the Trans Am NF-κB p65 ELISA kit according to the manufacturers protocol. The colorimetric readout was quantified using a spectrophotometer (Ultrospec®3000 UV/Visible Spectrophotometer (Pharmacia Biotech)) at 450 nm with a reference wavelength of 655 nm.

## 2.8. Immunohistochemistry (IHC) on cultured porcine EC

Porcine EC were grown as monolayers on glass coverslips (VWR) placed in a 6 well plate. After 48 hours incubation the cells were stimulated with 0, 15 or 30 minutes TNF-α (10 ng/ml). Alternatively porcine EC were grown on glass slides (VWR) and after 48 hours of incubation the cells were exposed to 0, 30 or 90 minutes of flow.

### 2.8.1. Staining for p65

After treatment (with TNFα or flow) the slides/coverslips were rinsed briefly with PBS. The PBS was aspirated and 4% paraformaldehyde was applied for 10 minutes to fixate the cells. After fixation, the cells were rinsed 3 times with PBS. The cells were then

permeabilized using 0.1% triton X100/PBS for 4 minutes. This stage was repeated before the slides/coverslips were rinsed 3 times using 0.5 % BSA/PBS. The cells were blocked for 30 minutes using 0.5 % BSA/PBS (blocking buffer).

The primary antibody (NF-κB p65 (C-20) rabbit polyclonal 100 μg/ml, Santa Cruz Biotechnology) was diluted 1:200 in the blocking buffer and added to the cells. The slides were incubated with the primary antibody for 1 hour, before the slides/coverslips were rinsed with blocking buffer twice and washed 3 times for 10 minutes also with the blocking buffer.

The cells were then incubated with the secondary antibody (Alexa Fluor 568 goat anti-rabbit 2mg/ml, Molecular Probes) (diluted 1:300 in blocking buffer) and a FITC-conjugated DBA lectin antibody (Fluorescein labeled Dolichos Biflorus Agglutinin, Vector Laboratories) (diluted 1:100 in blocking buffer) for 45 minutes. After this incubation the cells were rinsed in blocking buffer twice and washed 3 times for 10 minutes also in blocking buffer. Finally the slides/coverslips were rinsed once with PBS and once with sterile water.

The coverslips were mounted onto glass slides using Aqua Poly/Mount (Polysciences Inc). Similarly the coverslips were mounted onto the glass slides, and the slides were photographed and analyzed using a confocal LSM microscope (Zeiss LSM 510 META).

# 3. RESULTS

## 3.1. Validation of the phenotype of cultured cells

First we had to validate that the cells we isolated from porcine aortas and porcine jugular veins by collagenase treatment, are in fact endothelial cells. To achieve this we performed phase contrast microscopy, immuno fluorescence confocal microscopy and flow cytometry. Phase contrast microscopy revealed that both PAEC and PJVEC assume a "cobble stone" morphology in culture (Figure 3.1, upper panel). The cells could be stained with FITC DBA lectin (Figure 3.1, centre panel) with a positive marker of EC. It is known that endothelial cells express the surface protein CD31 (PECAM-1), smooth muscle cells however, does not express this protein. CD31 is therefore frequently used as a marker of endothelial cells. We observed by immunostaining and flow cytometry that >90 % PAEC and PJVEC expressed CD31 (Figure 3.1, lower panel). In conclusion, the PAEC and PJVEC cultures used in my study were indeed of endothelial origin.

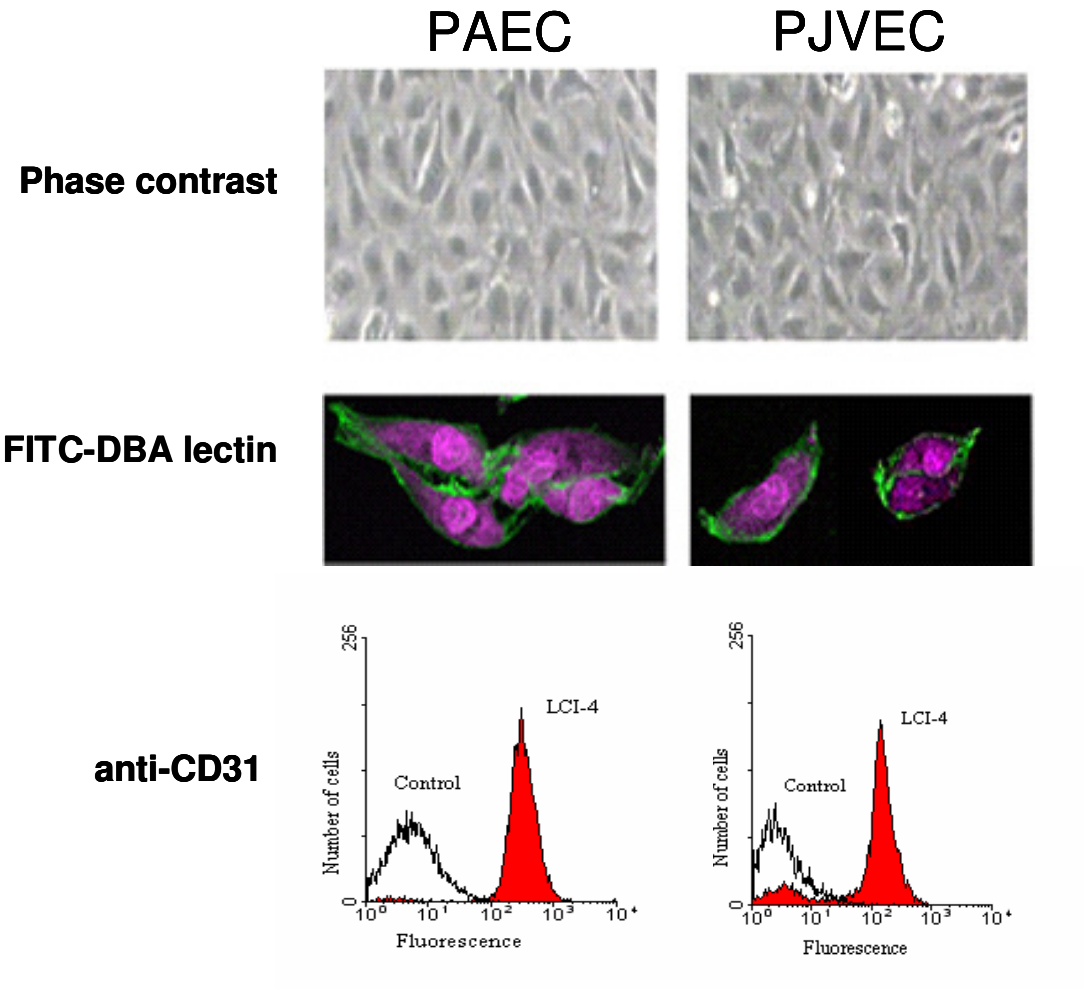


**Figure 3.1 Porcine EC.** Cells cultured on gelatin-coated coverslips were fixed with 4% paraformaldehyde for 15mins before analysis by phase contrast microscopy (upper panel). Alternatively, fixed cells were stained using FITC-conjugated DBA lectin (green) and counterstained with To-Pro 5 iodide to identify nuclei (pink) followed by laser-scanning confocal microscopy at X40 magnification (LSM 510 META, Zeiss) (middle panel). Porcine aortic endothelial cells (PAEC) were stained for CD31(PECAM-1) using LCI-4 primary antibodies and FITC-conjugated secondary antibodies before analysis by flow cytometry. Alternatively, cells were incubated with isotype-matched control antibodies (lower panel) (Data from M.Zakkar).

## 3.2. Endothelial activation in response to TNFα

We assessed pro-inflammatory activation of PAEC and PJVEC in response to TNFα (Figure 3.2, left upper panel). Neither of the populations expressed E-selectin or VCAM-1 constitutively. TNFα induced E-selectin surface expression in approximately 50 % of the PAEC following 4 hours treatment, which decreased to approximately 25 % at 24 hours. Similarly, TNFα induced E-selectin expression in approximately 60 % of the PJVEC at 4 hours and 25 % at 24 hours of treatment (Figure 3.2, right upper panel). In PAEC, TNFα induced VCAM-1 expression in approximately 70 % of the cells after 2 hours stimulation which decreased to approximately 60 % at 24 hours of treatment. TNFα induced VCAM-1 surface expression in approximately 70 % PJVECs at 2 both 2 and 24 hours of treatment.

I conclude that both PAEC and PJVEC express E-selectin and VCAM-1 in response to TNFα and that the kinetics of the response is broadly similar between the cell types.

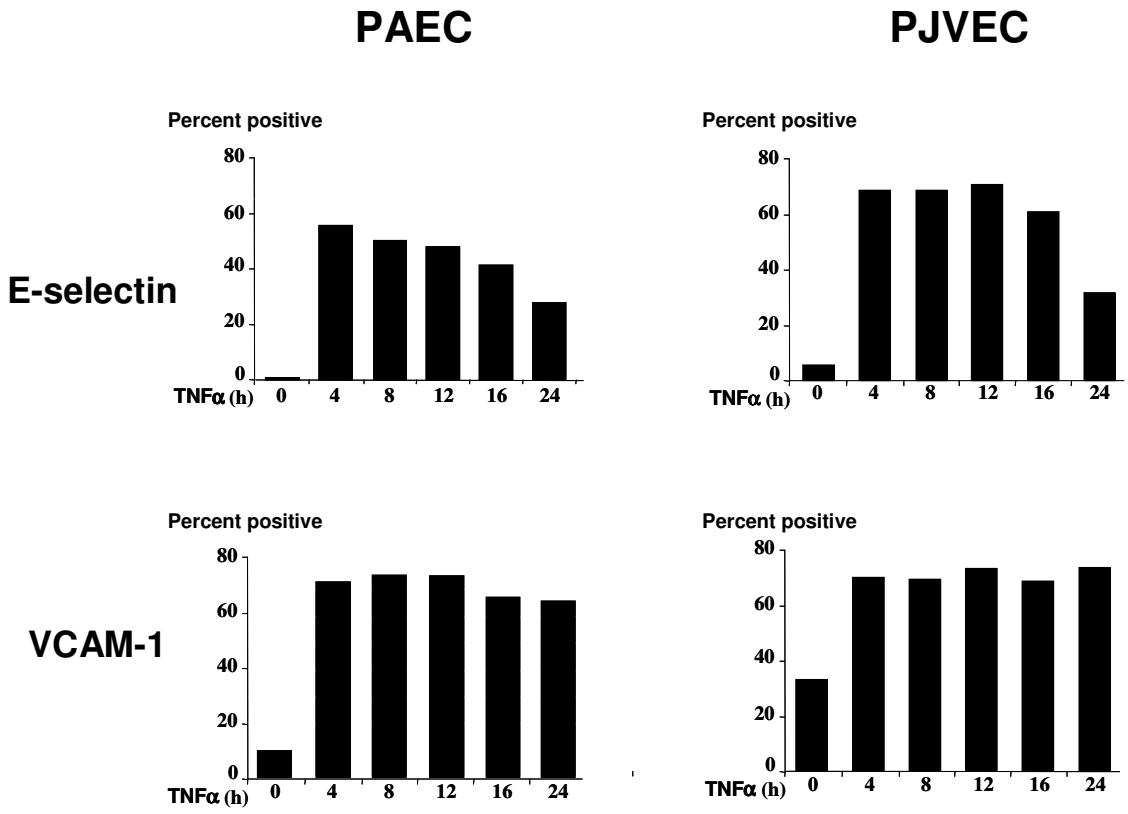


**Figure 3.2 Porcine EC in response to TNFα** Flow cytometry Cultured cells were stimulated with TNFα (10ng/ml) and, surface expression of E-selectin or VCAM-1 was quantified by immunostaining using anti-E-selectin (mAb 1.2B6) or anti-VCAM-1 antibodies (mAb 10.2C7) and FITC-conjugated secondary antibodies followed by flow cytometry. Positive cells were identified as those with greater fluorescence than cells incubated with isotype-matched control antibodies/FITC-conjugated secondary. Values represent the percentage of positive cells at each time point. (Data from M.Zakkar)

## 3.3. Acute laminar flow (12 dynes/cm$^2$) induces inflammatory chemokines and adhesion molecules

My initial experiments were on cultured monolayers of PAEC and PJVEC. The cells were plated on gelatin coated glass slides for 48 hours before they were either mounted in a vacuum sealed parallel flow chamber system or left under static conditions in an incubator (37 ˚C, 5% $CO_2$, 95% $O_2$). The cells were exposed to either 0, 2 or 4 hours of laminar flow with 12 dynes/cm$^2$ shear stress. After 0, 2 or 4 hours of flow the cells were briefly rinsed in cold PBS before the cells were lysed and total RNA was extracted. cDNA was synthesized using the mRNA extracts, and we used this cDNA to run comparative RT-PCR looking firstly at pro-inflammatory genes. Porcine versions of IL-8, MCP-1, IP-10, E-selectin, P-selectin, VCAM-1, ICAM-1 and cyclophilin primers were designed. The transcript levels were normalised to the housekeeping gene cyclophilin.

### 3.3.1. Chemokine expression

We measured transcript levels of three chemokines: IL-8, MCP-1 and IP-10 in static cells or cells exposed to shear stress. Our experiments show that MCP-1 and IL-8 are significantly upregulated at the mRNA level in PJVEC exposed to arterial flow for 2 and 4 hours (Figure 3.3, right side upper and centre panels). In contrast 2 hour or 4 hour laminar flow had little or no effect on IL-8 or MCP-1 in PAEC (Figure 3.3, left side upper and centre panels). This is an interesting difference between the porcine aortic EC and the porcine jugular vein EC which suggest that the PJVECs are hypersensitive to acute shear stress. IP-10 transcript levels remain relatively unchanged in PJVEC after

flow treatment for both 2 and 4 hours (Figure 3.3, lower right panel). In PAEC our data show that 2 hours of shear stress downregulate the transcription of IP-10, while at 4 hours of flow the transcript level is increasing towards the basal level (Figure 3.3, lower left panel).

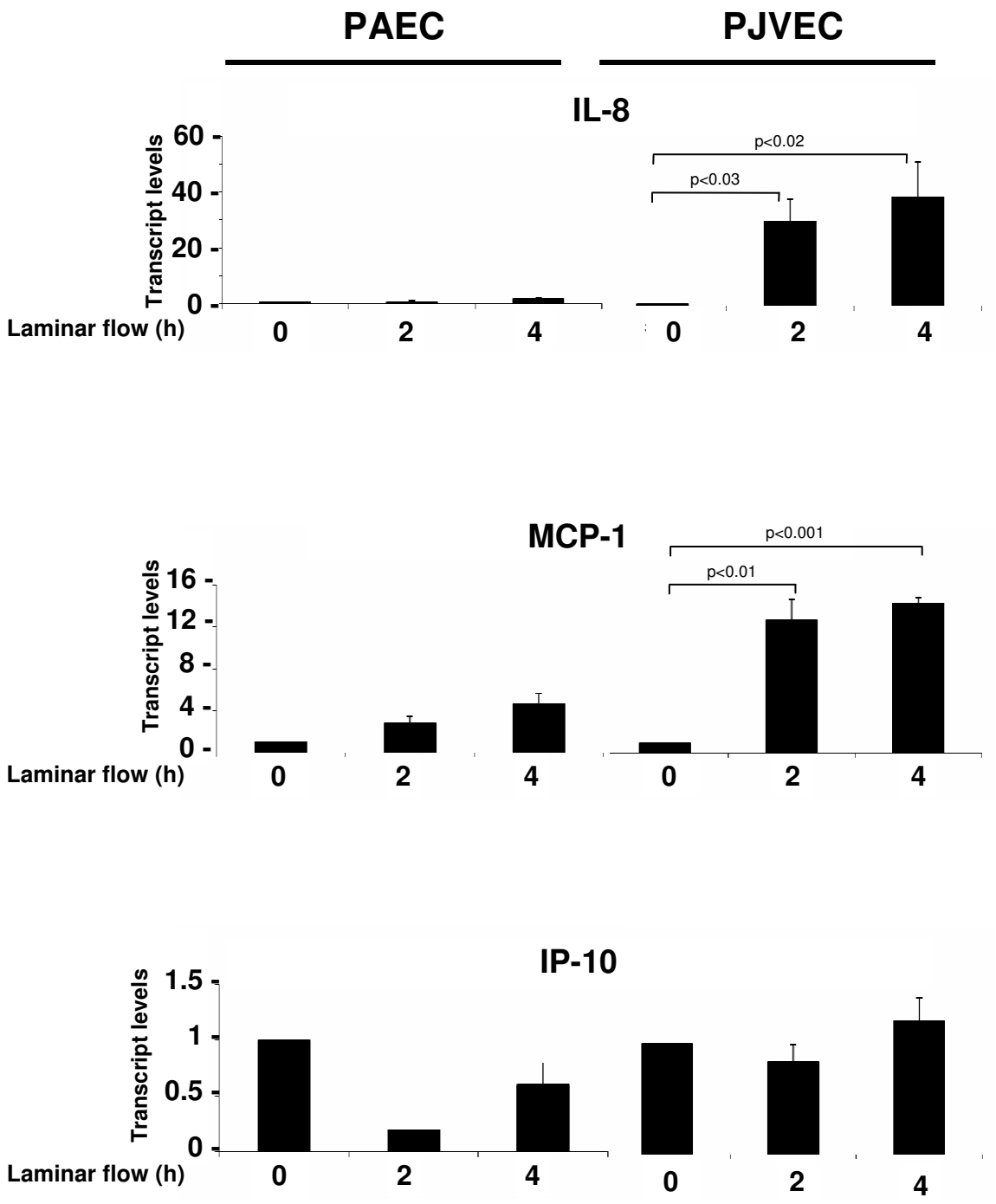


**Figure 3.3 Chemokine induction.** PAECs and PJVEC were cultured on glass slides for 48 hours before they were mounted onto a laminar flow chamber and exposed to flow for the indicated times. Quantitative RT-PCR was performed to analyze relative amounts of the genes. The transcript levels shown are normalised to the housekeeping gene cyclophilin and all the samples are normalised to the static control (0 hours flow) (n=3).

### 3.3.2. Adhesion molecule expression

Next I examined the expression of two selectins; E-selectin and P-selectin, and two other adhesion molecules; VCAM-1 and ICAM-1 in flow-conditioned EC.

Our data show that E-selectin is transiently upregulated by acute flow in PJVEC, with a peak at 2 hours (3.5-fold) of shear stress (Figure 3.4, upper right panel). Comparatively, E-selectin seems to be slightly downregulated by acute laminar flow in PAEC (Figure 3.4, upper left panel).

P-selectin is down regulated in both PAEC and PJVEC by acute shear stress (Figure 3.4, lower panels).

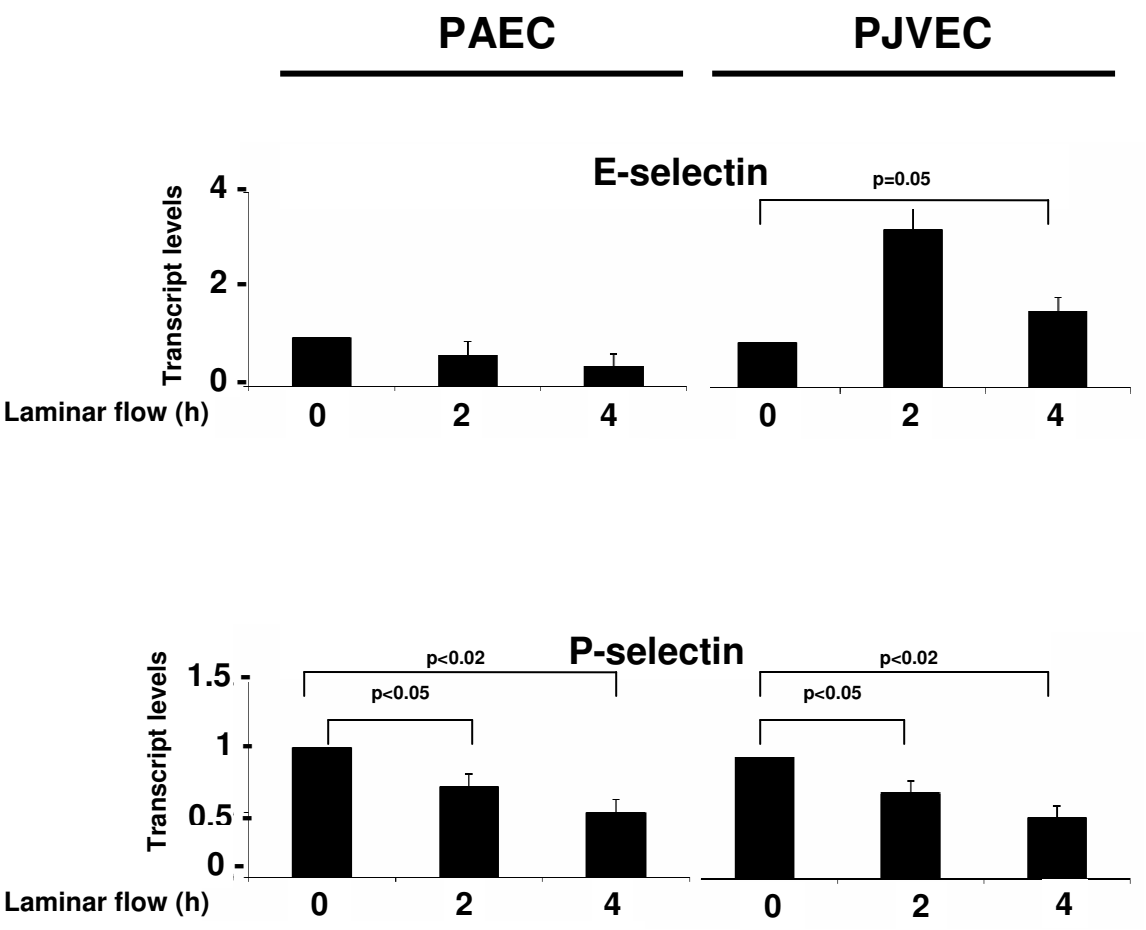


**Figure 3.4 Transcription of selectins in response to flow.** PAEC and PJVEC were cultured on glass slides for 48 hours before they were mounted onto a laminar flow chamber and exposed to flow for the indicated times. Quantitative RT-PCR was performed to analyze relative amounts of the genes. The transcript levels are normalised to the housekeeping gene cyclophilin and all the samples are normalised to the static control (0 hours flow) (n=3). Statistics were done using paired student's t-test.

Our experiments suggest that neither VCAM-1 (Figure 3.5, upper panel) nor ICAM-1 (Figure 3.5, lower panel) mRNA levels are upregulated by acute laminar flow in either PAEC or PJVEC.

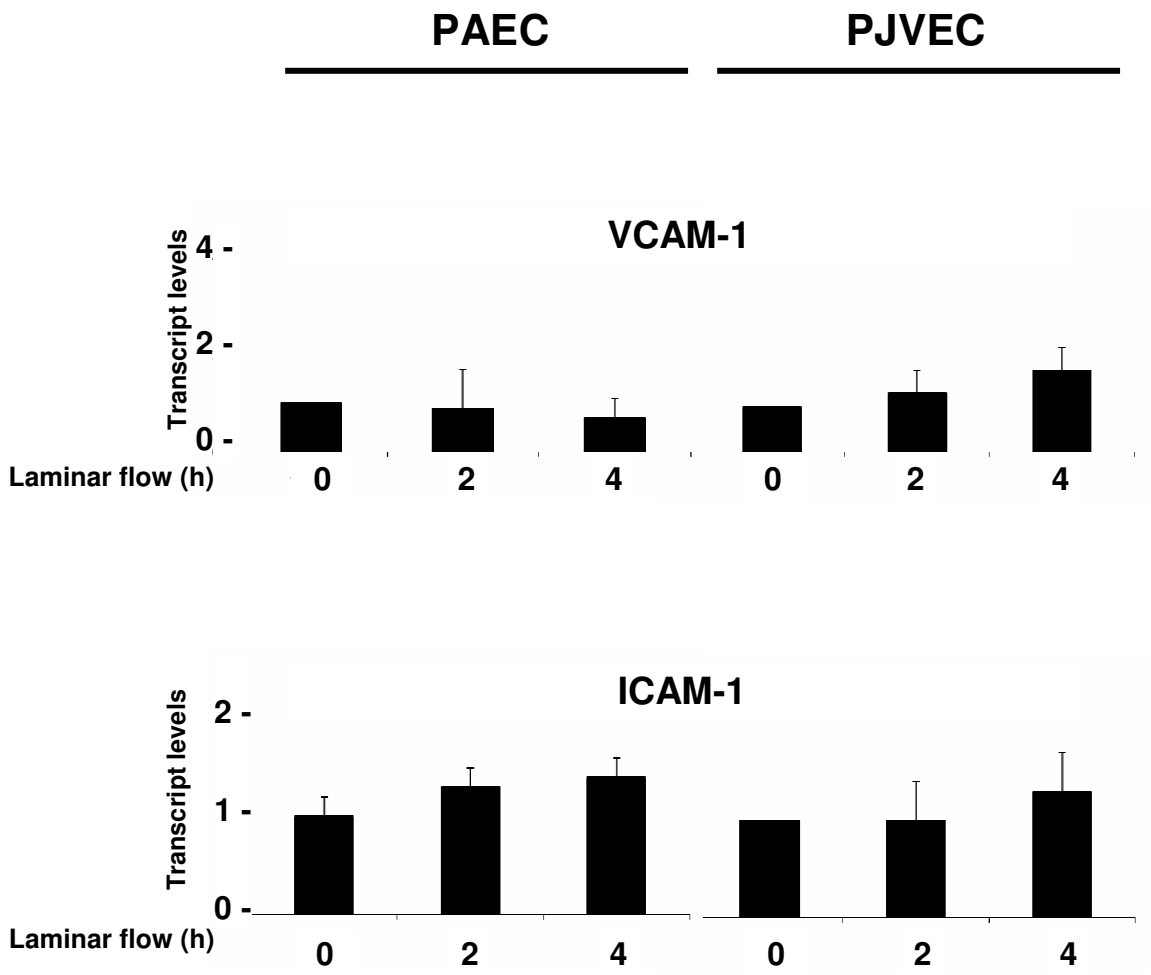


**Figure 3.5 Adhesion molecule transcripts.** PAEC and PJVEC were cultured on glass slides for 48 hours before they were mounted onto a laminar flow chamber and exposed to flow for the indicated times. Quantitative RT-PCR was performed to analyze relative amounts of the genes. The transcript levels are normalised to the housekeeping gene cyclophilin and all the samples are normalised to the static control (0 hours flow) (n=3).

## 3.4. MAP-kinase activation

We suspected that the differences in gene expression in PAEC and PJVECs in response to acute laminar flow might be due to differences in the level of activation of the MAP-kinase and the NF-κB pathways. To investigate the underlying mechanisms of the gene expression we focused on the p38 and JNK MAPK pathways and finally we looked at NF-κB.

### 3.4.1. p38

To assess whether acute flow altered the level of p38 activation in PAEC and PJVEC we performed western blot analysis on protein lysates obtained from flow experiments where we used the Cytodyne parallel flow chamber loop. The cells were cultured on glass slides as previously shown, for 48 hours. The cells were then exposed to either static conditions or laminar flow for 30 or 90 minutes. TNFα (10 ng/ml) treatment was used as the positive control on slides under static conditions. Our group routinely use the primary antibodies (total p38 and phosphorylated p38 (New England Biolabs)), and they are known to specifically recognize all the p38 isoforms.

Western blotting revealed that acute flow did not alter p38 activation in PAEC (Figure 3.6, left hand panels). However in PJVEC we observed that p38 was activated in response to flow (Figure 3.6, right panel). This increase in p38 MAPK activation in PJVEC, although modest, may be an important factor in explaining PJVEC hypersensitivity to acute laminar flow at arterial shear. Because this experiment was only done once for the

PJVEC and twice for the PAEC this needs to be investigated further before firm conclusions may be drawn.

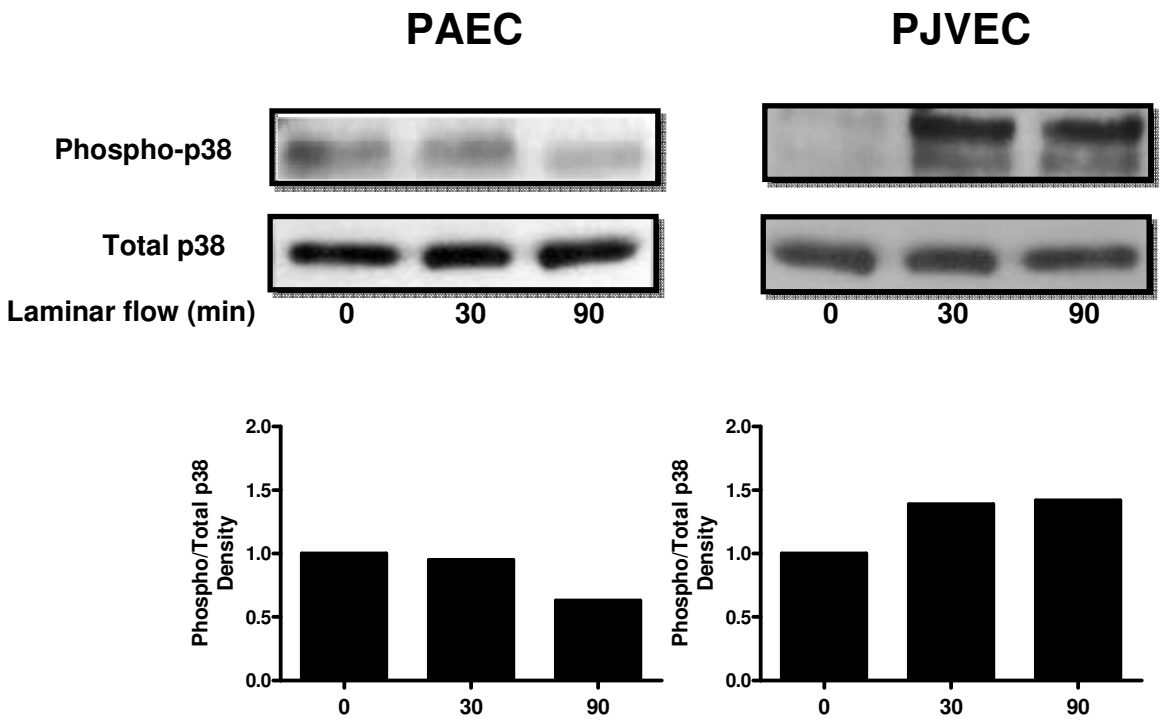


**Figure 3.6 p38 activation ito flow.** The cells were cultured as monolayers on glass slides for 48 hours before they were exposed to laminar flow for the indicated times. The cells were lysed using the NucBuster kit and the protein concentration for each sample was calculated using a UV spechtrophotometer. We added equal amounts of protein to each well and the blots were developed using Western Lightning chemoluminescense (upper panel). The densitometry was calculated using Image-J software and the y-axis is showing the phosphorylated p38 / total p38 ratio (lower panel).

### 3.4.2. JNK

As with the p38 MAPK experiments we performed western blots on protein lysates from cells exposed to laminar flow or static contidions to assess the kinetics of JNK activation in cultured PAEC and PJVEC. The JNK antibodies (total and phosphorylated JNK, both from New England Biolabs) are known to specifically bind to JNK-1/2 (46 kDa and 54 kDa) protein. We then applied the densitometry technique (Image-J software) to quantify the activation.

JNK is transiently activated by acute laminar flow in PAEC with a peak at 30 minutes (Figure 3.7, left panel). However in PJVEC, JNK activation by arterial flow is prolonged when compared to the PAECs and the peak is at 90 minutes (Figure 3.7, right panel). This is an interesting observation that may explain the induction of pro inflammatory transcripts in PJVEC in response to acute laminar flow at arterial shear.

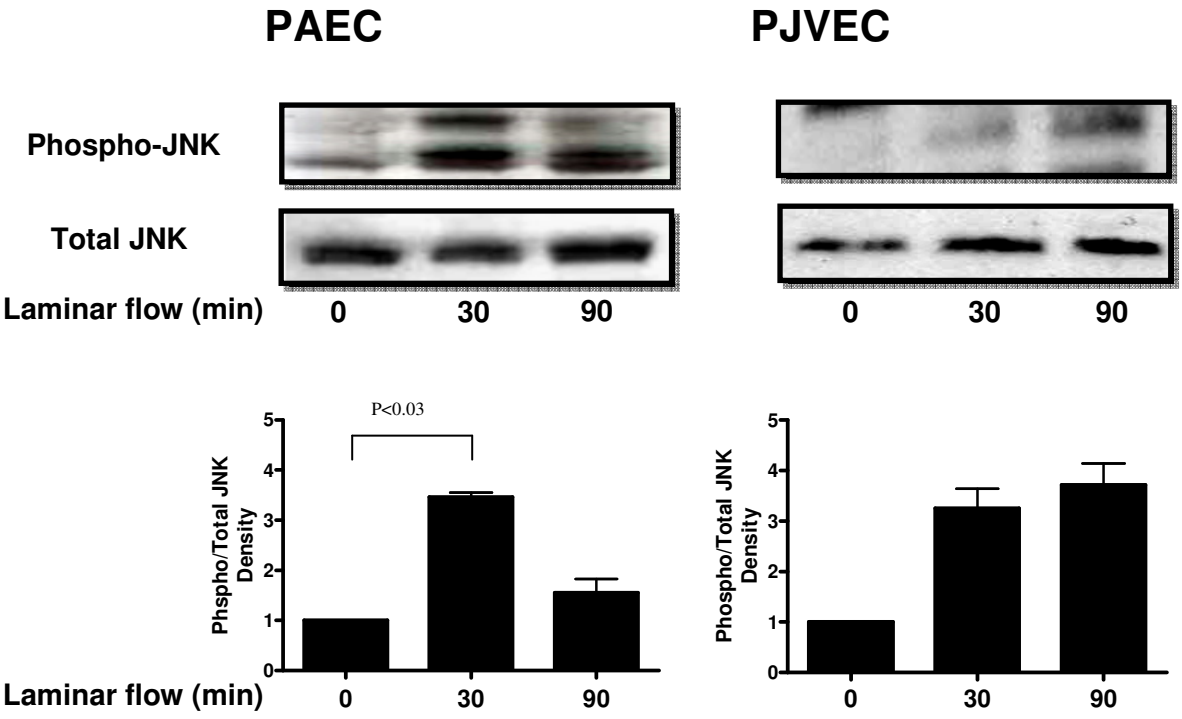


**Figure 3.7 JNK activation in response to flow.** The cells were cultured as monolayers on glass slides for 48 hours before they were exposed to laminar flow for the indicated times. The cells were lysed using the NucBuster kit and the protein concentration for each sample was calculated using a UV spechtrophotometer. We added equal amounts of protein to each well and the blots were developed using Western Lightning chemoluminescense (upper panel). The densitometry was calculated using Image-J software and the y-axis is showing the phospho JNK / total JNK ratio (lower panel) (n=2) Statistics done using a paired student's t-test.

## 3.5. NF-κB p65

I investigated NF-κB activation in response to acute shear stress using monolayers of PAEC and PJVEC plated onto glass slides. Initially I performed western blotting on nuclear lysates using anti-p65 or anti-lamin B antibodies (anti-lamin B antibody served as a loading control for nuclear proteins).

My data revealed that p65 translocated to the nucleus after approximately 30 minutes of laminar flow and was increased further at 90 minutes in PAEC (Figure 3.8.A, left panel). Western blotting of PJVEC lysates gave complicated results. On face value, it appears that p65 is present in nuclear lysates for static and flow-treated PJVEC. However, the levels of lamin B (nuclear protein) are very low. This suggests that the lysates may be "contaminated" with high levels of cytosolic material. Due to these technical difficulties it is impossible to draw firm conclusions from this experiment. Thus we approached the question of p65 activation in porcine EC in a different manner, by applying the immunohistochemistry (IHC) technique and analyze the cells by confocal laser-scanning microscopy (LSM) (section 3.5.1 and 3.5.2 below).

To confirm the p65 nuclear translocation in PAEC in response to laminar flow we also performed an ELISA quantification with a kit from Trans Am (Figure 3.8.B, left panel). We used nuclear lysates obtained in the same manner as the lysates for the previously shown western blots (Nucbuster). Due to before mentioned complications in culturing PJVECs, we did not have any protein lysate available to do these experiments for the venous endothelial cells.

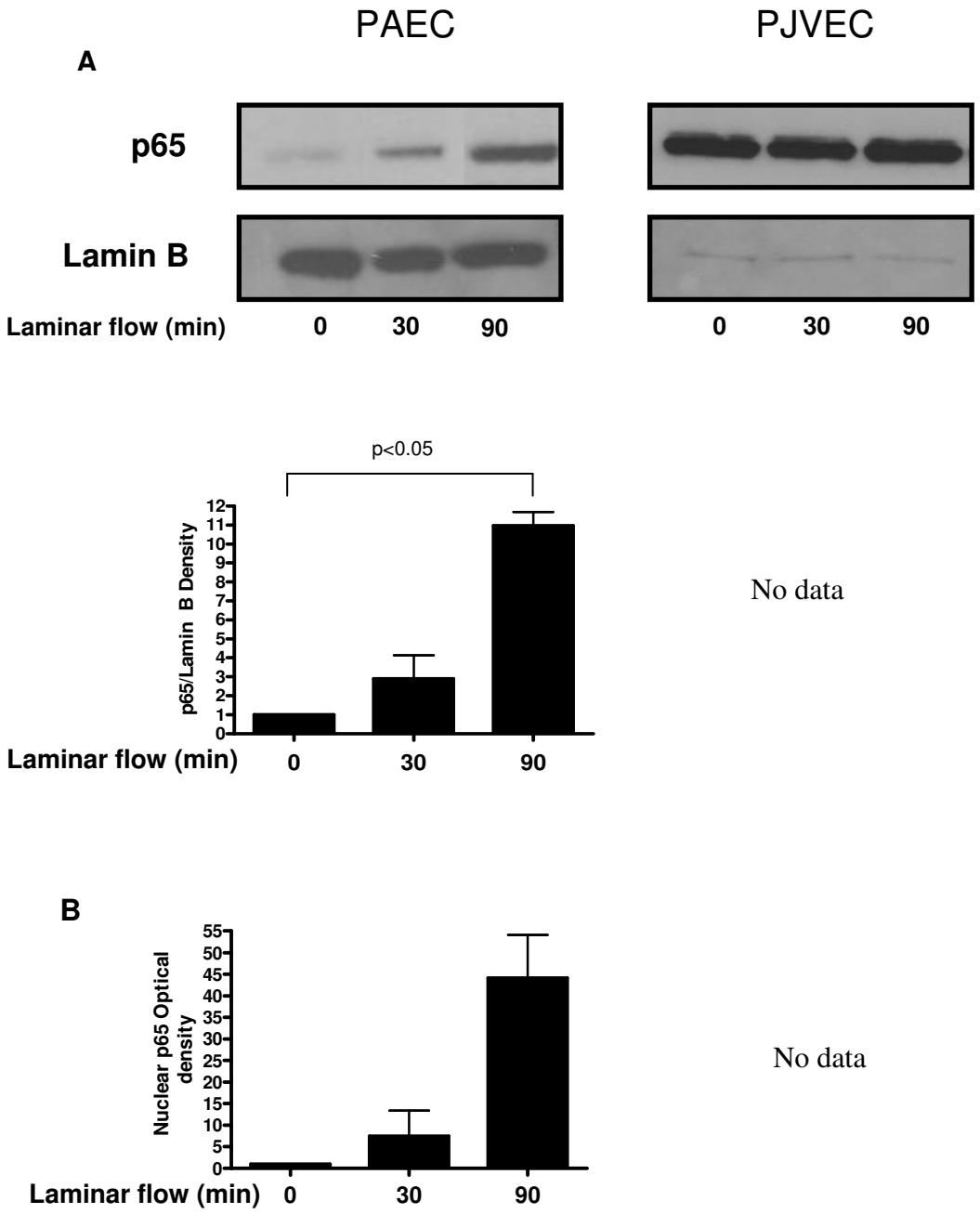


**Figure 3.8 NF-κB activation in response to flow.** The cells were cultured as monolayers on glass slides for 48 hours before they were exposed to laminar flow for the indicated times. The cells were lysed using the NucBuster kit and the protein concentration for each sample was calculated using a UV spechtrophotometer. The nuclear fraction of the protein lysate was used in the western blot analysis and the constitutive nuclear protein lamin B was used as a loading control. The blots were developed using Western Lightning chemoluminescense. The densitometry was calculated using Image-J software and the y-axis is showing the p65/lamin B ratio (n=2) (A). Alternatively TransAm ELISA kit was used to quantify nuclear p65. The optical density was measured in a UV spectrophotometer (n=2) (B) Statistics were done using paired student's t-test.

### 3.5.1. p65 response to TNFα using immunohistochemistry

Cells were plated on glass slides and left for 48 hours before they were treated with flow for 0, 30 and 90 minutes. We then fixed the cells in 4 % paraformaldehyde and proceeded with the staining protocol. We wanted to look at the NF-κB activation and thus stained for p65. We included the FITC-conjugated DBA as a marker for endothelium. The slides were then analyzed by confocal LSM microscopy.

I assessed the response of PAEC and PJVEC to TNF-α to ensure that the technique was adequate. In ustimulated cells, p65 was localised to the cytoplasm in both PAEC and PJVEC (Figure 3.9). This supports our previous PAEC data (western blots and ELISAs) and our suspicion that the PJVEC western blot analysis was flawed. Upon 15-30 minutes of stimulation with TNFα, p65 clearly concentrates within the oval shaped nucleus of the PAEC and PJVEC (Figure 3.9; compare centre and lower panels with upper panels). Thus p65 translocates to the nucleus in both cell types in response to TNFα.

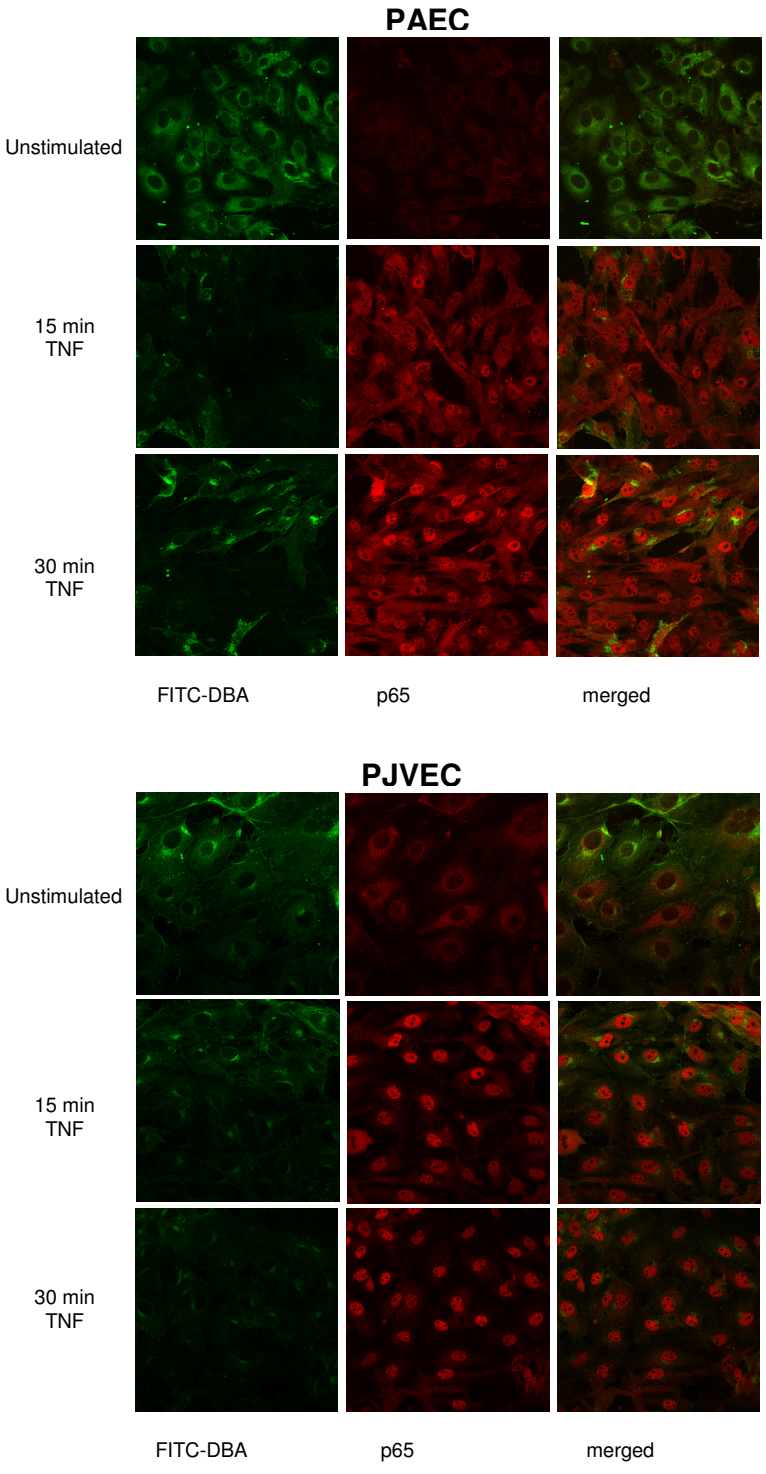


**Figure 3.9 TNFα induces p65 translocation in porcine EC.** Confocal LSM microscopy (LSM 510 META, Zeiss, 40X magnification). The cells were cultured as monolayers on glass slides for 48 hours before they were stimulated with TNF-α for the indicated times. Then the cells were stained using rabbit anti- p65 primary antibody (Santa Cruz) and UV fluorescent 568nm anti-rabbit secondary antibody (red) and FITC-conjugated DBA antibody (green) (n=1).

### 3.5.2. p65 response to laminar flow

We next examined the p65 response in PAEC and PJVEC exposed to laminar flow.

Upon exposure to laminar flow (12 dynes $cm^2$) for 30 minutes, p65 was partly located in the nucleus and partly in the cytoplasm in both PAEC and PJVEC (Figure 3.10; compare centre and lower panels with upper panels). This is consistent with the previous data for PAEC (western blots and ELISAs (Figure 3.8.A and 3.8.B) which indicated that NF-κB can be activated by flow in these cells). After 90 minutes of laminar flow, p65 was further concentrated in the nucleus of PJVEC. Unfortunately we do not have data for PAEC exposed to laminar flow for 90 minutes, because of technical complications in this experiment. In summary, acute laminar flow triggered nuclear localisation of NF-κB in both PAEC and PJVEC. Put together, our data therefore imply that PAEC and PJVEC respond in a similar manner to acute laminar flow in the respect of p65 activation.

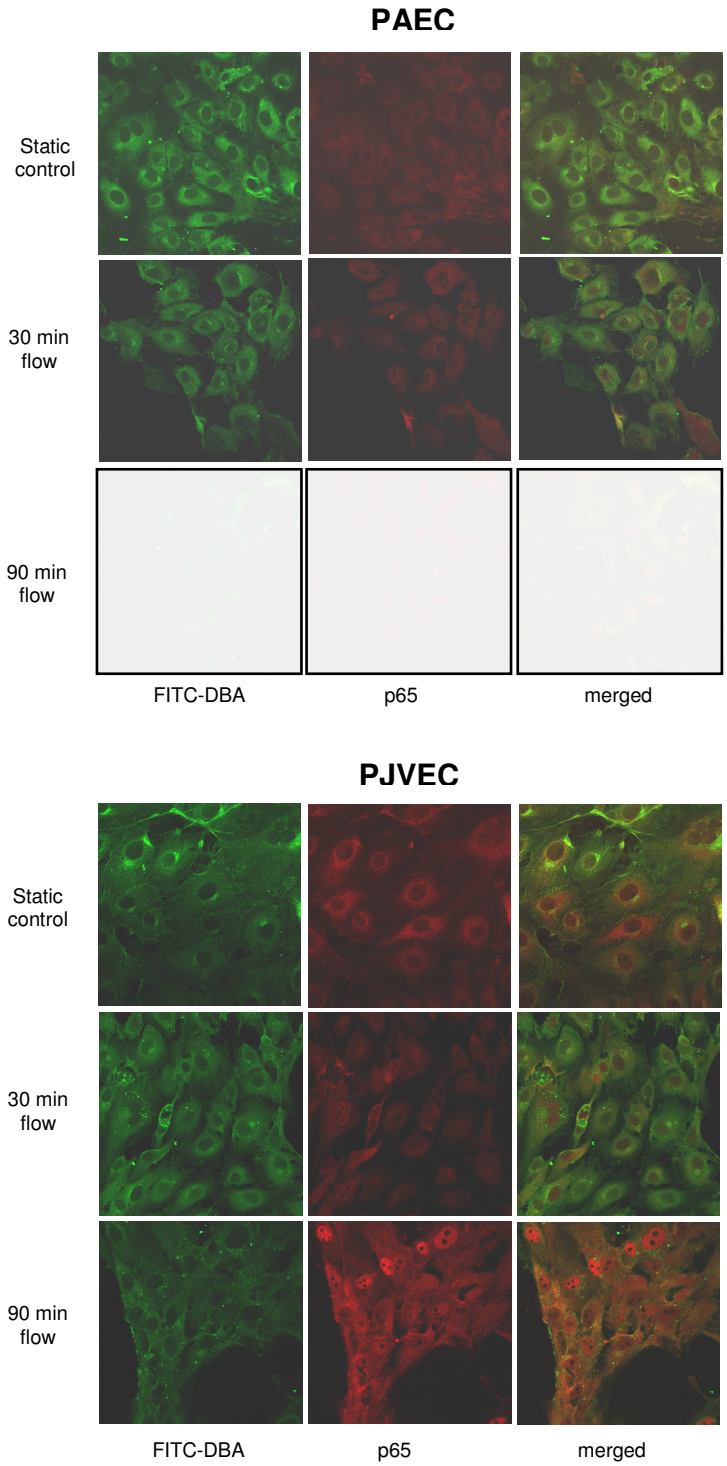


**Figure 3.10 Flow induces p65 translocation in porcine EC.** Confocal LSM microscopy (LSM 510 META, Zeiss, 40X magnification). The cells were cultured as monolayers on glass slides for 48 hours before they were treated with laminar flow in the Cytodyne flow loop for the indicated times. Then the cells were stained using rabbit anti- p65 primary antibody (Santa Cruz) and UV fluorescent 568nm anti-rabbit secondary antibody (red) and FITC-conjugated DBA antibody (green) (n=1).

## 3.6. Cytoprotective and anti-inflammatory transcription is induced by acute flow

Our experiments suggest that PJVEC show increased activation of MAPKs and elevated transcription of pro-inflammatory genes in response to laminar flow compared to PAEC (Figure 3.3 and Figure 3.4). We hypothesised that PAEC may be more resistant to the pro-inflammatory effects of acute laminar flow compared to PJVEC due to elevated expression of negative regulators of inflammation such as MKP-1, Cezanne, XIAP, Keap-1, Nrf2 and A20. Comparative real time PCR was used to assess the level of each of these transcripts in static EC, cells exposed to laminar flow for 4 hours and in cells exposed to TNF-α for 2 hours (as a positive control for expression) (Figure 3.11 and Figure 3.12)

We observed considerable variation between experiments in the expression levels of several molecules in static PAEC and in flow treated or TNFα treated cells. The reasons for this are unclear but it is plausible that they may relate to differences in the confluency, passage number or genetics of the primary EC used. Nevertheless, several reproducible observations could be made which are described below.

For PAEC the experiments were performed 3 times (n=3), but for the PJVEC the experiments was only done once (n=1) due to complications encountered in culturing the cells during the later stages of the project.

### 3.6.1. Quantitative RT PCR

Our real time PCR data suggest that MKP-1 transcripts were upregulated approximately 8-fold by acute laminar flow in PAECs, but not in PJVECs (Figure 3.11, upper panel).

An interesting observation in our data is that Cezanne mRNA seems to be unaffected by acute laminar flow in PAEC, but is suppressed by flow in PJVECs (Figure 3.11, centre panel).

I observed that acute laminar flow induced XIAP by approximately 2-fold in PAEC but did not alter the expression of XIAP in PJVEC (Figure 3.11, lower panel). By contrast, TNFα induced XIAP in both PAEC and PJVEC (Figure 3.11, lower panel).

Furthermore, we could not observe any reproducible changes in the expression of Keap-1, Nrf2 or A20 in response to acute laminar flow in either PAEC or PJVEC (Figure 3.12).

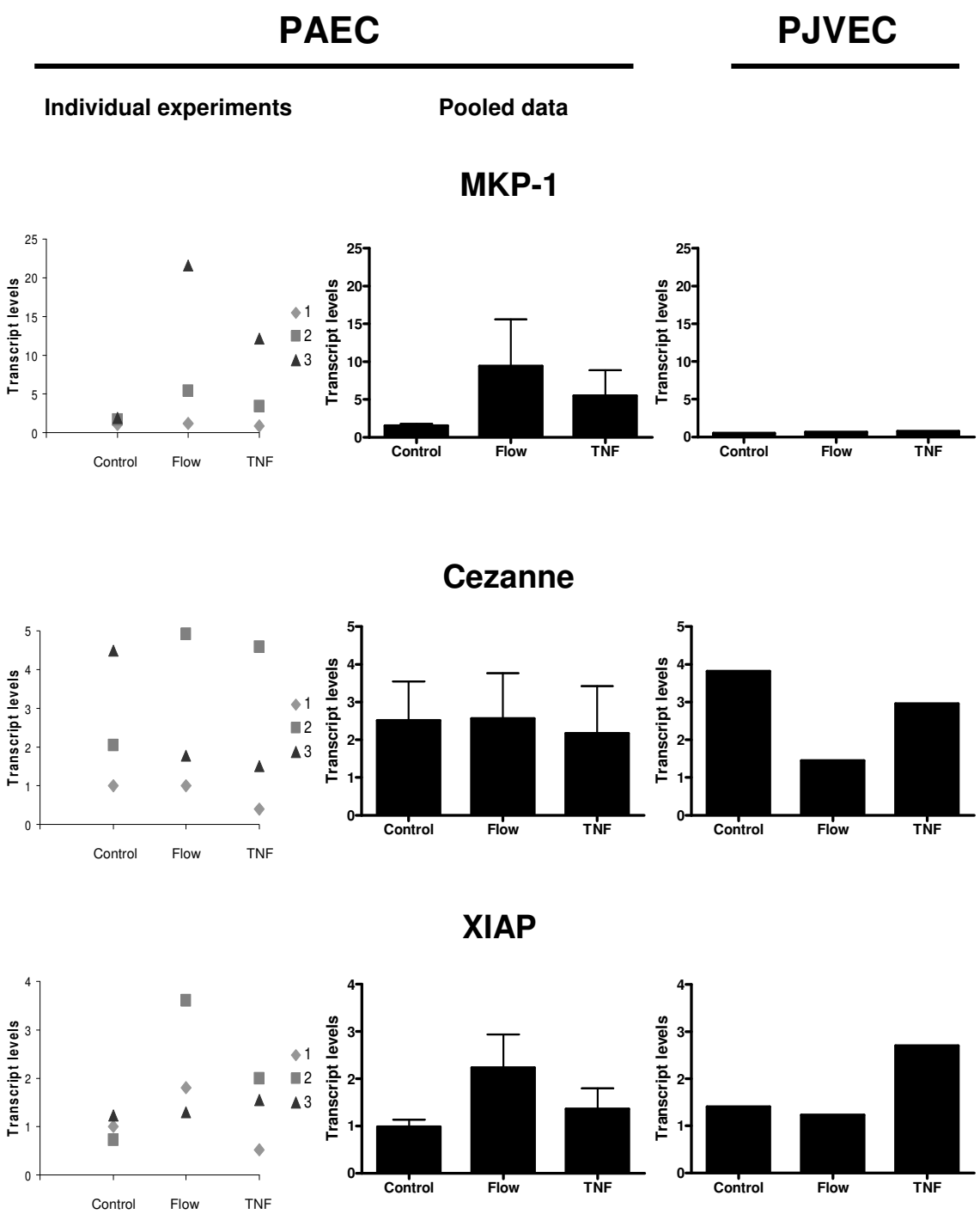


**Figure 3.11 Induction of cytoprotective genes in porcine EC.** PAEC and PJVEC were cultured on glass slides for 48 hours before they were mounted onto a laminar flow chamber and exposed to flow for 4 hours, kept in static conditions or stimulated with TNFα (10 ng/ml) for 2 hours. RT-PCR was performed to analyze relative amounts of the genes. The transcript levels were normalised to the housekeeping gene cyclophilin, and all the samples are normalised to the PAEC control sample 1. The experiments were repeated 3 times for the PAECs and once for PJVEC. Because of extensive variation in the PAEC samples the variation is shown in the left column.

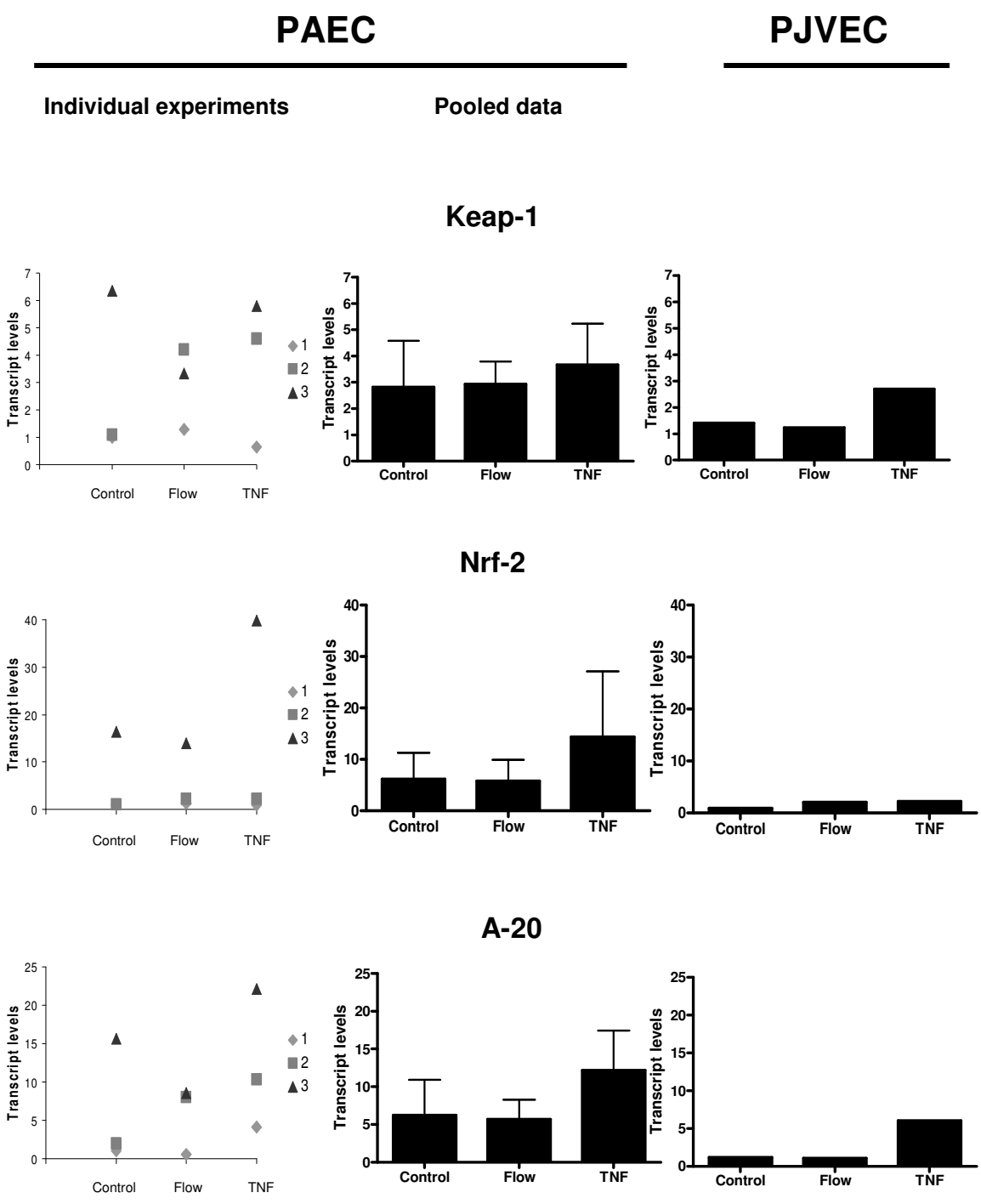


**Figure 3.12 Induction of cytoprotective transcripts in porcine EC.** PAEC and PJVEC were cultured on glass slides for 48 hours before they were mounted onto a laminar flow chamber and exposed to flow for 4 hours, kept in static conditions or stimulated with TNFα (10 ng/ml) for 2 hours. Quantitative RT-PCR was performed to analyze relative amounts of genes. The transcript levels were normalised to the housekeeping gene cyclophilin, and all the samples are normalised to the PAEC control sample 1. The experiments were repeated 3 times for the PAECs and once for PJVECs. Because of extensive variation in the PAEC samples the variation is shown in the left column.

Thus my preliminary data suggests that the anti-inflammatory molecules MKP-1 and XIAP are upregulated in response to flow in PAEC but not in PJVEC. This may be important in explaining the PAEC comparative resistance to pro-inflammatory activation in response acute shear stress.

Further studies are now required to determine whether the preliminary data that I generated is genuinely representative of the transcriptional response of PAEC and PJVEC to acute laminar flow.

# 4. DISCUSSION

## 4.1. Induction of pro-inflammatory transcripts

We showed that acute laminar flow at arterial shear (12 dynes/cm$^2$) significantly upregulated the transcription of the pro-inflammatory chemokines MCP-1 and IL-8 as well as the adhesion molecule E-selectin in PJVEC but only had modest effects on PAEC.

The differences in MCP-1 and IL-8 transcription in response to acute flow strongly suggest that venous EC may have an increased tendency to become activated and recruit inflammatory cells in response to shear stress compared to aortic EC. It is possible that this initial inflammatory response may lay the foundation for intimal hyperplasia and subsequent atherosclerosis which in turn leads to graft failure (see section 1.4.4. for details).

Other groups have previously shown that VCAM-1 and ICAM-1 transcript levels are upregulated by laminar flow (Burns and DePaola, 2005; Morigi *et al.*, 1995a; Morigi *et al.*, 1995b; Nagel *et al.*, 1994), however, we did not see this in our experiments. This may be explained by the fact that the other groups looked at 6 hour time point of flow exposure, while we looked at 2 and 4 hours of flow exposure.

## 4.2. Pro-inflammatory signalling pathways and their regulation

### 4.2.1. p38

In my western blotting experiments we showed that acute flow did not alter the activation of the MAPK p38 in PAEC. However, in PJVEC p38 activation might be upregulated. This needs to be confirmed, due to the fact that this experiment only was done once. The reason for this was because of the shortage of PJVEC protein lysate, which in turn was a result of the complications we encountered in culturing the PJVEC. We were planning to repeat the experiment for the PAEC, but we did not have time towards the end of the project. It is therefore important for members of our group to now repeat this experiment before we can draw strong conclusions about the differences in p38 activation in PAEC and PJVEC. It is interesting to note however, that evidence showing that p38 is essential in regulating MCP-1 expression in EC (Goebeler *et al.*, 1999) strenghtens our hypothesis that p38 is in fact activated to a lesser extent in PAEC than in PJVEC in response to acute flow.

### 4.2.2. JNK

As seen in the results section 3.4.2, JNK is transiently upregulated in response to acute flow in PAEC. However, in PJVEC the JNK activation is prolonged, which leads to increased transcription of AP1-dependent genes, such as MCP-1, IL-8 and E-selectin (Hoefen and Berk, 2002). This correlates well with our results showing the increased transcription of the pro-inflammatory genes in PJVEC exposed to laminar flow. It would be interesting to include further time points in our study to further investigate the kinetics

of JNK activation. Because the experiments were done only twice we would have to do at least one more repeat to draw strong conclusions.

I analysed EC protein lysates for JNK acivation by ELISA as well (data not shown) with a kit from BioSource. However, the results were impossible to determine because none of the samples gave a signal above the background signal. This might be because the antibodies used were not compatible with porcine versions of JNK, although the manufacturer of the kit claims they are. Another possible reason is that the Nucbuster lysis buffer used for protein extraction is not compatible with the ELISA kit from BioSource.

Previous studies have shown that the E-selectin promoter has an ATF2-c-Jun sequence in addition to NF-κB binding sites (Wadgaonkar *et al.*, 2004). The increased E-selectin transcription in PJVEC correlates with our findings that the JNK and p38 activation is extended in PJVEC compared to PAEC. In addition, it is thought that all the MAPKs might under some circumstances act as cytoprotective or antiapoptotic signalling molecules. For instance, it has recently been shown that p38 is important in cytoskeletal remodelling in response to fluid shear stress, which is an important atheroprotective force (Azuma *et al.* 2001).

I have only considered the activation of p38 and JNK MAP kinases in this project. It is therefore important to investigate the ERK and BMK1 pathways as well, as there is suggested that there are complex crosstalk between the pro-inflammatory and

cytoprotective pathways. Because the pro-inflammatory MAPKs (p38 and JNK) and the cytoprotective MAPKs (ERK and BMK1) have contradictory roles, the cellular response is regulated by relative activation of the pro-inflammatory and cytoprotective MAPKs p38 (Hoefen and Berk, 2002) (Figure 4.1).

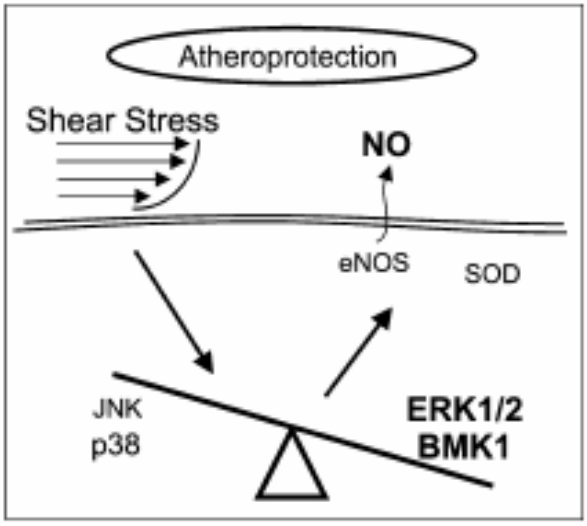


**Figure 4.1 MAPK involvement in endothelial activation** Continous high laminar shear stress may activate ERK1/2 and BMK1 to a higher extent than p38 and JNK which wouldlead to atheroprotection (Hoefen and Berk, 2002)

### 4.2.3. p65

I initially wanted to investigate p65 activation in porcine EC in response to flow by using the western blot technique. While this technique worked well with the PAEC, we had complications with the protein lysates obtained from the cultured PJVEC. As mentioned in the results section 3.5, the nuclear lysates seemed to be contaminated with cytosolic material. This might be due to lysis buffer degeneration. No matter the cause, these particular protein lysates were not usable for further experiments. Because of the "contaminated" protein lysated from the PJVECs we could not perform densitometry on

the westerns or employ the ELISA kit technique. As a result we had to investigate p65 activation in PJVEC in response to flow by other means. We decided to do this by employing immunohistochemistry (IHC) and the confocal laser-scanning microscopy (LSM). This technique worked well with both cell types, and using IHC we found conclusively that p65 is located in the cytoplasm of quiescent (control) PAEC and PJVEC.

Furthermore, the data suggests that laminar flow triggers transient nuclear localisation of p65 which occurred with similar kinetics in PAEC and PJVEC. However, further experiments are needed to confirm these data.

## 4.3. Transcription of anti-inflammatory genes

As mentioned in the results section (3.6.1) I saw considerable variations in the transcription of the anti-inflammatory genes. The reason for this is unclear but may be a result of differences in confluency, passage number or in the genetics of the primary EC. One “marker” commonly used for indicating the capacity of EC in responce to flow is transcription of HO-1 (heme-oxygenase-1), ie. HO-1 is expected to be significantly upregulated at transcriptional level by flow. In future studies we should therefore use this as a control for EC consistency.

My data suggest that MKP-1 and XIAP may be upregulated in response to acute laminar flow in PAEC but not in PJVEC. This is an important observation which suggests that PAEC may be able to restrict the initial inflammatory response to flow by limiting the

phosphorylation of p38 and JNK through induction of MKP-1 and XIAP (Figure 4.2). However, these findings are preliminary and needs to be addressed further.

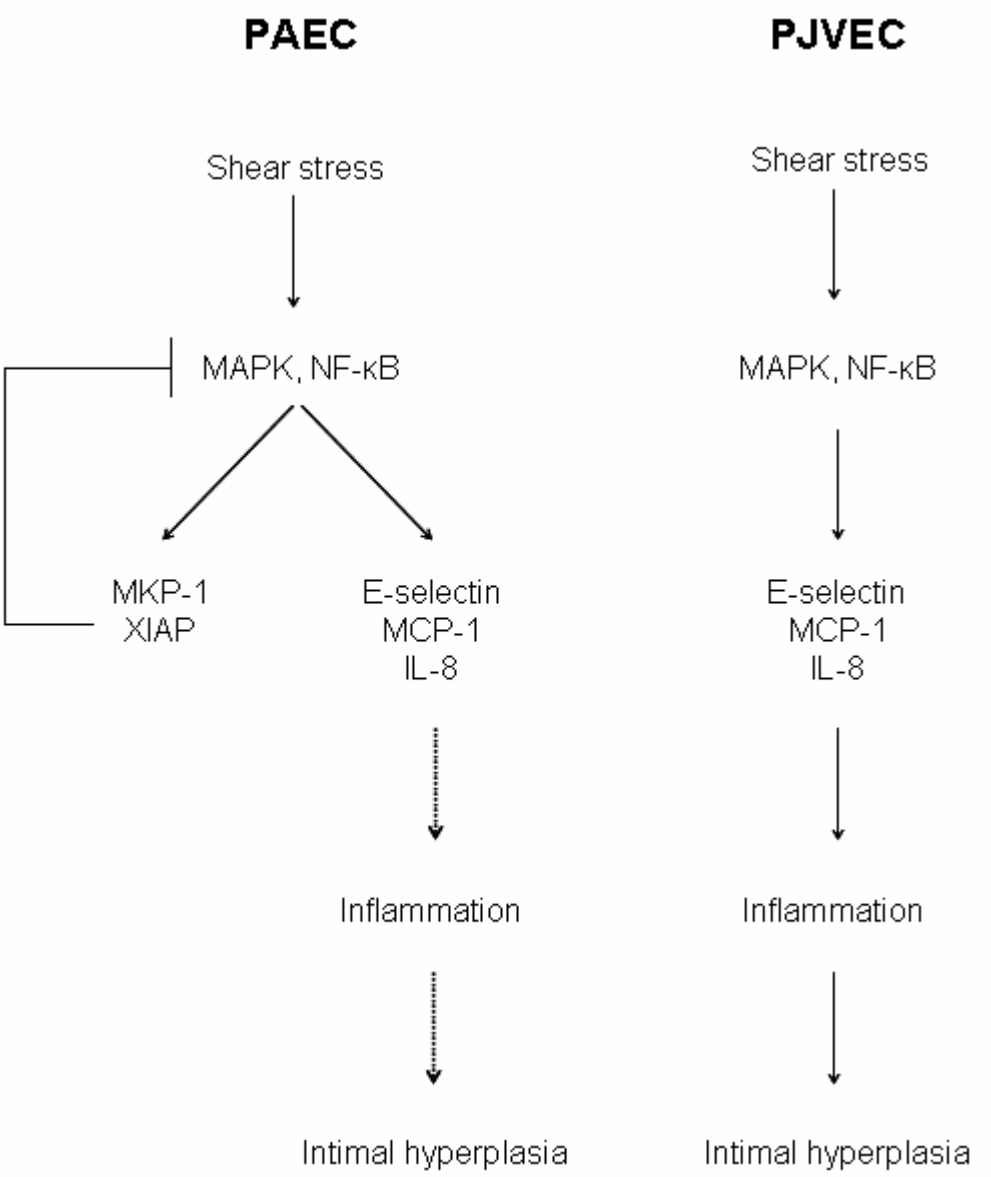


**Figure 4.2 Model for restriction of inflammation in PAEC.** We suggest this model for restriction of inflammation (and further intimal hyperplasia and graft faliure) in PAEC by induction of MKP-1and XIAP transcripts in response to laminar flow.

Regarding the other anti-inflammatory/cytoprotective genes studied, the variation is too extensive to draw any firm conclusions. Further studies are therefore required.

## 4.4. Future studies and clinical relevance

It would be interesting to examine whether specific blocking of p38, JNK and/or NF-κB leads to inhibition of MCP-1, IL-8 and E-selectin transcription in EC. We were planning to investigate this as a part of this project, but as time ran out for me, other members of our group will continue with this investigation using novel pharmacological inhibitors of p38 and JNK.

From a clinical point of view, my findings may be of relevance in coronary artery bypass grafting. In particular, my study raises the possibility that pre-treating vein grafts with inhibitors of MAPK and NF-κB before transpositioning them into arterial circulation may reduce inflammation and therefore improve their patency.

# 5. CONCLUSIONS

- Arterial shear stress activates cultured porcine jugular vein endothelial cells (PJVEC).

- Acute laminar flow induced high levels of IL-8, MCP-1 and E-selectin transcripts in PJVEC.

- By contrast, arterial EC were relatively resistant to the pro-inflammatory effects of shear stress.

- The induction of pro-inflammatory mRNA is accompanied by induction of cytoprotective transcripts (MKP-1 and XIAP) in PAEC, but not in PJVEC.

- The hypersensitivity of venous EC to flow-mediated pro-inflammatory changes may rely on the prolonged (>90mins) activation of JNK and p38 MAP kinases.

- The hypersensitivity of venous EC to the pro-inflammatory effects of shear stress may partly explain the susceptibility of vein grafts to inflammation and accelerated atherosclerosis.

# 6. REFERENCES

## 6.1. Reference list


Ahmad, M., P. Theofanidis, and R. M. Medford. 1998. Role of activating protein-1 in the regulation of the vascular cell adhesion molecule-1 gene expression by tumor necrosis factor-alpha. *The Journal of Biological Chemistry* 273, (8) (Feb 20): 4616-4621.

Amano, J., A. Suzuki, M. Sunamori, T. Tsukada, and F. Numano. 1991. Cytokinetic study of aortocoronary bypass vein grafts in place for less than six months. *The American Journal of Cardiology* 67, (15) (Jun 1): 1234-1236.

Anwar, A. A., F. Y. Li, D. S. Leake, T. Ishii, G. E. Mann, and R. C. Siow. 2005. Induction of heme oxygenase 1 by moderately oxidized low-density lipoproteins in human vascular smooth muscle cells: Role of mitogen-activated protein kinases and Nrf2. *Free Radical Biology & Medicine* 39, (2) (Jul 15): 227-236.

Arenzana-Seisdedos, F., P. Turpin, M. Rodriguez, D. Thomas, R. T. Hay, J. L. Virelizier, and C. Dargemont. 1997. Nuclear localization of I kappa B alpha promotes active transport of NF-kappa B from the nucleus to the cytoplasm. *Journal of Cell Science* 110 ( Pt 3), (Pt 3) (Feb): 369-378.

Azuma, N., N. Akasaka, H. Kito, M. Ikeda, V. Gahtan, T. Sasajima, and B. E. Sumpio. 2001. Role of p38 MAP kinase in endothelial cell alignment induced by fluid shear stress. *American Journal of Physiology.Heart and Circulatory Physiology* 280, (1) (Jan): H189-97.

Bhullar, I. S., Y. S. Li, H. Miao, E. Zandi, M. Kim, J. Y. Shyy, and S. Chien. 1998. Fluid shear stress activation of IkappaB kinase is integrin-dependent. *The Journal of Biological Chemistry* 273, (46) (Nov 13): 30544-30549.

Burns, M. P., and N. DePaola. 2005. Flow-conditioned HUVECs support clustered leukocyte adhesion by coexpressing ICAM-1 and E-selectin. *American Journal of Physiology.Heart and Circulatory Physiology* 288, (1) (Jan): H194-204.

Bustin, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* 25, (2) (Oct): 169-193.

Cornelissen, J., J. Armstrong, and C. M. Holt. 2004. Mechanical stretch induces phosphorylation of p38-MAPK and apoptosis in human saphenous vein. *Arteriosclerosis, Thrombosis, and Vascular Biology* 24, (3) (Mar): 451-456.

Cunningham, K. S., and A. I. Gotlieb. 2005. The role of shear stress in the pathogenesis of atherosclerosis. *Laboratory Investigation; a Journal of Technical Methods and Pathology* 85, (1) (Jan): 9-23.

Dai, G., M. R. Kaazempur-Mofrad, S. Natarajan, Y. Zhang, S. Vaughn, B. R. Blackman, R. D. Kamm, G. Garcia-Cardena, and M. A. Gimbrone Jr. 2004. Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and -resistant regions of human vasculature. *Proceedings of the National Academy of Sciences of the United States of America* 101, (41) (Oct 12): 14871-14876.

Davies, P. F. 2007. Endothelial mechanisms of flow-mediated athero-protection and susceptibility. *Circulation Research* 101, (1) (Jul 6): 10-12.

Davis, M. E., I. M. Grumbach, T. Fukai, A. Cutchins, and D. G. Harrison. 2004. Shear stress regulates endothelial nitric-oxide synthase promoter activity through nuclear factor kappaB binding. *The Journal of Biological Chemistry* 279, (1) (Jan 2): 163-168.

Davis, R. J. 2000. Signal transduction by the JNK group of MAP kinases. *Cell* 103, (2) (Oct 13): 239-252.

Dimmeler, S., J. Haendeler, V. Rippmann, M. Nehls, and A. M. Zeiher. 1996. Shear stress inhibits apoptosis of human endothelial cells. *FEBS Letters* 399, (1-2) (Dec 9): 71-74.

Dong, C., R. J. Davis, and R. A. Flavell. 2002. MAP kinases in the immune response. *Annual Review of Immunology* 20, : 55-72.

Dozois, C. M., E. Oswald, N. Gautier, J. P. Serthelon, J. M. Fairbrother, and I. P. Oswald. 1997. A reverse transcription-polymerase chain reaction method to analyze porcine cytokine gene expression. *Veterinary Immunology and Immunopathology* 58, (3-4) (Sep 19): 287-300.

Evans, P. C., T. S. Smith, M. J. Lai, M. G. Williams, D. F. Burke, K. Heyninck, M. M. Kreike, R. Beyaert, T. L. Blundell, and P. J. Kilshaw. 2003. A novel type of deubiquitinating enzyme. *The Journal of Biological Chemistry* 278, (25) (Jun 20): 23180-23186.

Evans, P. C., E. R. Taylor, J. Coadwell, K. Heyninck, R. Beyaert, and P. J. Kilshaw. 2001. Isolation and characterization of two novel A20-like proteins. *The Biochemical Journal* 357, (Pt 3) (Aug 1): 617-623.

Gardner, T. J. 2007. Searching for the second-best coronary artery bypass graft: Is it the radial artery? *Circulation* 115, (6) (Feb 13): 678-680.

Ghosh, S., M. J. May, and E. B. Kopp. 1998. NF-kappa B and rel proteins: Evolutionarily conserved mediators of immune responses. *Annual Review of Immunology* 16, : 225-260.

Gibson, U. E., C. A. Heid, and P. M. Williams. 1996. A novel method for real time quantitative RT-PCR. *Genome Research* 6, (10) (Oct): 995-1001.

Goebeler, M., K. Kilian, R. Gillitzer, M. Kunz, T. Yoshimura, E. B. Brocker, U. R. Rapp, and S. Ludwig. 1999. The MKK6/p38 stress kinase cascade is critical for tumor necrosis factor-alpha-induced expression of monocyte-chemoattractant protein-1 in endothelial cells. *Blood* 93, (3) (Feb 1): 857-865.

Gonzales, R. S., and T. M. Wick. 1996. Hemodynamic modulation of monocytic cell adherence to vascular endothelium. *Annals of Biomedical Engineering* 24, (3) (May-Jun): 382-393.

Grabowski, E. F., A. J. Reininger, P. G. Petteruti, O. Tsukurov, and R. W. Orkin. 2001. Shear stress decreases endothelial cell tissue factor activity by augmenting secretion of tissue factor pathway inhibitor. *Arteriosclerosis, Thrombosis, and Vascular Biology* 21, (1) (Jan): 157-162.

Guan, Z., S. Y. Buckman, A. P. Pentland, D. J. Templeton, and A. R. Morrison. 1998. Induction of cyclooxygenase-2 by the activated MEKK1 --> SEK1/MKK4 --> p38 mitogen-activated protein kinase pathway. *The Journal of Biological Chemistry* 273, (21) (May 22): 12901-12908.

Hansson, G. K., and G. P. Berne. 2004. Atherosclerosis and the immune system. *Acta Paediatrica (Oslo, Norway : 1992).Supplement* 93, (446) (Dec): 63-69.

Hansson, G. K., P. Libby, U. Schonbeck, and Z. Q. Yan. 2002. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circulation Research* 91, (4) (Aug 23): 281-291.

Hay, D. C., C. Beers, V. Cameron, L. Thomson, F. W. Flitney, and R. T. Hay. 2003. Activation of NF-kappaB nuclear transcription factor by flow in human endothelial cells. *Biochimica Et Biophysica Acta* 1642, (1-2) (Sep 23): 33-44.

Herlaar, E., and Z. Brown. 1999. p38 MAPK signalling cascades in inflammatory disease. *Molecular Medicine Today* 5, (10) (Oct): 439-447.

Hirai, S., M. Katoh, M. Terada, J. M. Kyriakis, L. I. Zon, A. Rana, J. Avruch, and S. Ohno. 1997. MST/MLK2, a member of the mixed lineage kinase family, directly phosphorylates and activates SEK1, an activator of c-jun N-terminal kinase/stress-activated protein kinase. *The Journal of Biological Chemistry* 272, (24) (Jun 13): 15167-15173.

Hoch, J. R., V. K. Stark, D. A. Hullett, and W. D. Turnipseed. 1994. Vein graft intimal hyperplasia: Leukocytes and cytokine gene expression. *Surgery* 116, (2) (Aug): 463-70; discussion 470-1.

Hoch, J. R., V. K. Stark, N. van Rooijen, J. L. Kim, M. P. Nutt, and T. F. Warner. 1999. Macrophage depletion alters vein graft intimal hyperplasia. *Surgery* 126, (2) (Aug): 428-437.

Hoefen, R. J., and B. C. Berk. 2002. The role of MAP kinases in endothelial activation. *Vascular Pharmacology* 38, (5) (May): 271-273.

Huang, T. T., N. Kudo, M. Yoshida, and S. Miyamoto. 2000. A nuclear export signal in the N-terminal regulatory domain of IkappaBalpha controls cytoplasmic localization of inactive NF-kappaB/IkappaBalpha complexes. *Proceedings of the National Academy of Sciences of the United States of America* 97, (3) (Feb 1): 1014-1019.

Ichijo, H., E. Nishida, K. Irie, P. ten Dijke, M. Saitoh, T. Moriguchi, M. Takagi, K. Matsumoto, K. Miyazono, and Y. Gotoh. 1997. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science (New York, N.Y.)* 275, (5296) (Jan 3): 90-94.

Ip, Y. T., and R. J. Davis. 1998. Signal transduction by the c-jun N-terminal kinase (JNK)--from inflammation to development. *Current Opinion in Cell Biology* 10, (2) (Apr): 205-219.

Jaattela, M., H. Mouritzen, F. Elling, and L. Bastholm. 1996. A20 zinc finger protein inhibits TNF and IL-1 signaling. *Journal of Immunology (Baltimore, Md.: 1950)* 156, (3) (Feb 1): 1166-1173.

Karin, M. 1999. The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. *The Journal of Biological Chemistry* 274, (39) (Sep 24): 27339-27342.

Krikos, A., C. D. Laherty, and V. M. Dixit. 1992. Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappa B elements. *The Journal of Biological Chemistry* 267, (25) (Sep 5): 17971-17976.

Kumar, S., J. Boehm, and J. C. Lee. 2003. p38 MAP kinases: Key signalling molecules as therapeutic targets for inflammatory diseases. *Nature Reviews.Drug Discovery* 2, (9) (Sep): 717-726.

Kwei, S., G. Stavrakis, M. Takahas, G. Taylor, M. J. Folkman, M. A. Gimbrone Jr, and G. Garcia-Cardena. 2004. Early adaptive responses of the vascular wall during venous arterialization in mice. *The American Journal of Pathology* 164, (1) (Jan): 81-89.

Lan, Q., K. O. Mercurius, and P. F. Davies. 1994. Stimulation of transcription factors NF kappa B and AP1 in endothelial cells subjected to shear stress. *Biochemical and Biophysical Research Communications* 201, (2) (Jun 15): 950-956.

Li, Y. S., J. Y. Shyy, S. Li, J. Lee, B. Su, M. Karin, and S. Chien. 1996. The ras-JNK pathway is involved in shear-induced gene expression. *Molecular and Cellular Biology* 16, (11) (Nov): 5947-5954.

Lin, K., P. P. Hsu, B. P. Chen, S. Yuan, S. Usami, J. Y. Shyy, Y. S. Li, and S. Chien. 2000. Molecular mechanism of endothelial growth arrest by laminar shear stress. *Proceedings of the National Academy of Sciences of the United States of America* 97, (17) (Aug 15): 9385-9389.

Mann, G. E., J. Niehueser-Saran, A. Watson, L. Gao, T. Ishii, P. de Winter, and R. C. Siow. 2007. Nrf2/ARE regulated antioxidant gene expression in endothelial and smooth muscle cells in oxidative stress: Implications for atherosclerosis and

preeclampsia. *Sheng Li Xue Bao [Acta Physiologica Sinica]* 59, (2) (Apr 25): 117-127.

Miao, H., S. Yuan, Y. Wang, A. Tsygankov, and S. Chien. 2002. Role of cbl in shear-activation of PI 3-kinase and JNK in endothelial cells. *Biochemical and Biophysical Research Communications* 292, (4) (Apr 12): 892-899.

Min, W., and J. S. Pober. 1997. TNF initiates E-selectin transcription in human endothelial cells through parallel TRAF-NF-kappa B and TRAF-RAC/CDC42-JNK-c-Jun/ATF2 pathways. *Journal of Immunology (Baltimore, Md.: 1950)* 159, (7) (Oct 1): 3508-3518.

Mitra, A. K., D. M. Gangahar, and D. K. Agrawal. 2006. Cellular, molecular and immunological mechanisms in the pathophysiology of vein graft intimal hyperplasia. *Immunology and Cell Biology* 84, (2) (Apr): 115-124.

Morigi, M., G. Micheletti, M. Figureliuzzi, B. Imberti, M. A. Karmali, A. Remuzzi, G. Remuzzi, and C. Zoja. 1995. Verotoxin-1 promotes leukocyte adhesion to cultured endothelial cells under physiologic flow conditions. *Blood* 86, (12) (Dec 15): 4553-4558.

Morigi, M., C. Zoja, M. Figureliuzzi, M. Foppolo, G. Micheletti, M. Bontempelli, M. Saronni, G. Remuzzi, and A. Remuzzi. 1995. Fluid shear stress modulates surface expression of adhesion molecules by endothelial cells. *Blood* 85, (7) (Apr 1): 1696-1703.

Moriguchi, T., N. Kuroyanagi, K. Yamaguchi, Y. Gotoh, K. Irie, T. Kano, K. Shirakabe, *et al.* 1996. A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *The Journal of Biological Chemistry* 271, (23) (Jun 7): 13675-13679.

Motwani, J. G., and E. J. Topol. 1998. Aortocoronary saphenous vein graft disease: Pathogenesis, predisposition, and prevention. *Circulation* 97, (9) (Mar 10): 916-931.

Nagel, T., N. Resnick, W. J. Atkinson, C. F. Dewey Jr, and M. A. Gimbrone Jr. 1994. Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells. *The Journal of Clinical Investigation* 94, (2) (Aug): 885-891.

Nakano, H. 2004. Signaling crosstalk between NF-kappaB and JNK. *Trends in Immunology* 25, (8) (Aug): 402-405.

Nakata, S., M. Tsutsui, H. Shimokawa, M. Tamura, H. Tasaki, T. Morishita, O. Suda, *et al.* 2005. Vascular neuronal NO synthase is selectively upregulated by platelet-derived growth factor: Involvement of the MEK/ERK pathway. *Arteriosclerosis, Thrombosis, and Vascular Biology* 25, (12) (Dec): 2502-2508.

Natarajan, R., S. Gupta, B. J. Fisher, S. Ghosh, and A. A. Fowler 3rd. 2001. Nitric oxide suppresses IL-8 transcription by inhibiting c-jun N-terminal kinase-induced AP-1 activation. *Experimental Cell Research* 266, (2) (Jun 10): 203-212.

Parhar, K., A. Ray, U. Steinbrecher, C. Nelson, and B. Salh. 2003. The p38 mitogen-activated protein kinase regulates interleukin-1beta-induced IL-8 expression via an effect on the IL-8 promoter in intestinal epithelial cells. *Immunology* 108, (4) (Apr): 502-512.

Pietersma, A., B. C. Tilly, M. Gaestel, N. de Jong, J. C. Lee, J. F. Koster, and W. Sluiter. 1997. p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. *Biochemical and Biophysical Research Communications* 230, (1) (Jan 3): 44-48.

Ratliff, N. B., and J. L. Myles. 1989. Rapidly progressive atherosclerosis in aortocoronary saphenous vein grafts. possible immune-mediated disease. *Archives of Pathology & Laboratory Medicine* 113, (7) (Jul): 772-776.

Read, M. A., M. Z. Whitley, S. Gupta, J. W. Pierce, J. Best, R. J. Davis, and T. Collins. 1997. Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. *The Journal of Biological Chemistry* 272, (5) (Jan 31): 2753-2761.

Rodriguez, M. S., J. Thompson, R. T. Hay, and C. Dargemont. 1999. Nuclear retention of IkappaBalpha protects it from signal-induced degradation and inhibits nuclear factor kappaB transcriptional activation. *The Journal of Biological Chemistry* 274, (13) (Mar 26): 9108-9115.

Ross, R. 1999. Atherosclerosis--an inflammatory disease. *The New England Journal of Medicine* 340, (2) (Jan 14): 115-126.

Saunders, P. C., G. Pintucci, C. S. Bizekis, R. Sharony, K. M. Hyman, F. Saponara, F. G. Baumann, *et al.* 2004. Vein graft arterialization causes differential activation of mitogen-activated protein kinases. *The Journal of Thoracic and Cardiovascular Surgery* 127, (5) (May): 1276-1284.

Sheikh, S., M. Rahman, Z. Gale, N. T. Luu, P. C. Stone, N. M. Matharu, G. E. Rainger, and G. B. Nash. 2005. Differing mechanisms of leukocyte recruitment and sensitivity to conditioning by shear stress for endothelial cells treated with tumour necrosis factor-alpha or interleukin-1beta. *British Journal of Pharmacology* 145, (8) (Aug): 1052-1061.

Sheikh, S., G. E. Rainger, Z. Gale, M. Rahman, and G. B. Nash. 2003. Exposure to fluid shear stress modulates the ability of endothelial cells to recruit neutrophils in response to tumor necrosis factor-alpha: A basis for local variations in vascular sensitivity to inflammation. *Blood* 102, (8) (Oct 15): 2828-2834.

Springer, T. A. 1995. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annual Review of Physiology* 57, : 827-872.

Stark, V. K., J. R. Hoch, T. F. Warner, and D. A. Hullett. 1997. Monocyte chemotactic protein-1 expression is associated with the development of vein graft intimal hyperplasia. *Arteriosclerosis, Thrombosis, and Vascular Biology* 17, (8) (Aug): 1614-1621.

Takekawa, M., F. Posas, and H. Saito. 1997. A human homolog of the yeast Ssk2/Ssk22 MAP kinase kinase kinases, MTK1, mediates stress-induced activation of the p38 and JNK pathways. *The EMBO Journal* 16, (16) (Aug 15): 4973-4982.

Ubeda, M., X. Z. Wang, H. Zinszner, I. Wu, J. F. Habener, and D. Ron. 1996. Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. *Molecular and Cellular Biology* 16, (4) (Apr): 1479-1489.

Wadgaonkar, R., J. W. Pierce, K. Somnay, R. L. Damico, M. T. Crow, T. Collins, and J. G. Garcia. 2004. Regulation of c-jun N-terminal kinase and p38 kinase pathways in endothelial cells. *American Journal of Respiratory Cell and Molecular Biology* 31, (4) (Oct): 423-431.

Weston, C. R., and R. J. Davis. 2007. The JNK signal transduction pathway. *Current Opinion in Cell Biology* 19, (2) (Apr): 142-149.

Whiteside, S. T., and A. Israel. 1997. I kappa B proteins: Structure, function and regulation. *Seminars in Cancer Biology* 8, (2) (Apr): 75-82.

Yamawaki, H., S. Lehoux, and B. C. Berk. 2003. Chronic physiological shear stress inhibits tumor necrosis factor-induced proinflammatory responses in rabbit aorta perfused ex vivo. *Circulation* 108, (13) (Sep 30): 1619-1625.

Zarubin, T., and J. Han. 2005. Activation and signaling of the p38 MAP kinase pathway. *Cell Research* 15, (1) (Jan): 11-18.

Zhang, L., K. Peppel, L. Brian, L. Chien, and N. J. Freedman. 2004. Vein graft neointimal hyperplasia is exacerbated by tumor necrosis factor receptor-1 signaling in graft-intrinsic cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* 24, (12) (Dec): 2277-2283.

Zou, Y., Y. Hu, M. Mayr, H. Dietrich, G. Wick, and Q. Xu. 2000. Reduced neointima hyperplasia of vein bypass grafts in intercellular adhesion molecule-1-deficient mice. *Circulation Research* 86, (4) (Mar 3): 434-440.

## 6.2. Internet references

1 **http://en.wikipedia.org/wiki/Image:Anatomy_artery.png**

2 **http://www.chemistry.sdsu.edu/graphics/huxford_nfkb_activation.jpg**

3 **http://www.merck.com/mmhe/print/sec03/ch033/ch033b.html**

4 **http://en.wikipedia.org/wiki/Image:PCR.svg**